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Title of invention: Bacterial Superantigen Vaccines

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APPLICATION FOR UNITED STATES PATENT

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INVENTION: Bacterial Superantigen Vaccines

SPECIFICATION

SAP 9/94

TITLE OF THE INVENTION

Bacterial Superantigen Vaccines

by
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Mark A. Olson
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INTRODUCTION

Staphylococcal enterotoxins (SEs) A through E are the most commom cause of food poisoning [Bergdoll, M.S.(1983) In Easom CSF, Aslam C., eds. Staphylococci and staphylococcal infections. London: Academic Press, pp 559-598] and are associated with several serious diseases [Schlievert, P.M. (1993) *J. Infect. Dis.* **167**: 997-1002; Ulrich *et al.* (1995) *Trends Microbiol.* **3**: 463-468], such as bacterial arthritis [Schwab *et al.* (1993) *J. Immunol.* **150**; 4151-4159; Goldenberg *et al.* (1985) *N. Engl. J. Med.* **312**: 764-771], other autoimmune disorders [Psnett, D. N. (1993) *Semin. Immunol.* **5**: 65-72], and toxic shock syndrome [Schlieverst, P.M. (1986) *Lancet* **1**: 1149-1150; Bohach *et al.* (1990) *Crit. Rev. Microbiol.* **17**: 251-272]. The nonenterotoxic staphylococcal superantigen toxic shock syndrome toxin-1 (TSST-1) was first identified as a causative agent of menstrual-associated toxic shock syndrome [Schlievert *et al.* (1981) *J. Infect. Dis.* **143**: 509-516]. Superantigen-producing *Staphylococcus aureus* strains are also linked to Kawasaki syndrome, an inflammatory disease of children [Leung *et al.* (1993) *Lancet* **342**: 1385-1388].

The staphylococcal enterotoxins A-E, toxic shock syndrome toxin-1 (TSST-1), and streptococcal pyrogenic

exotoxins A-C are soluble 23-29-kD proteins commonly referred to as bacterial superantigens (SAgs). Bacterial superantigens are ligands for both major histocompatibility complex (MHC) class II molecules, expressed on antigen-presenting cells, and the variable portion of the T cell antigen receptor β chain (TCR Vβ) [Choi *et al.* (1989) *Proc. Natl. Acad. Sci. USA* **86**:8941-8945; Fraser, J.D. (1989) *Nature* **339**:221-223; Marrack *et al.* (1990) *Science* **248**: 705-711; Herman *et al.* (1991) *Annu. Rev. Immunol.* **9**: 745-772; Mollick *et al.* (1989) *Science* **244**:817-820].

Each bacterial superantigen has a distinct affinity to a set of TCR Vβ, and coligation of the MHC class II molecule polyclonally stimulates T cells [White *et al.* (1989) *Cell* **56**: 27-35; Kappler *et al.* (1989) *Science* **244**: 811-813; Takimoto *et al.* (1990) *Eur J. Immunol.* **140**: 617-621]. Pathologically elevated levels of cytokines that are produced by activated T cells are the probable cause of toxic shock symptoms [Calson *et al.* (1985) *Cell. Immunol.* **96**: 175-183; Stiles *et al.* (1993) *Infect. Immun.* **61**: 5333-5338]. In addition, susceptibility to lethal gram-negative endotoxin shock is enhanced by several bacterial superantigens [Stiles, *et al.*, *supra*]. Although antibodies reactive with superantigens are present at low levels in human sera [Takei *et al.* (1993) *J. Clin. Invest.* **91**: 602-607], boosting antibody titers by specific immunization may be efficacious for patients at risk for toxic shock syndrome and the other disorders of common etiology.

A vaccine approach to controlling bacterial superantigen-associated diseases presents a unique set of challenges. Acute exposure to bacterial superantigens produces T cell anergy, a state of

specific non-responsiveness [Kawabe *et al.* (1991) *Nature* **349**: 245-248], yet T cell help is presumably a requirement for mounting an antibody response.

Presently, the only superantigen vaccines available are chemically inactivated toxoids from different bacteria which have several disadvantages. The chemical inactivation process can be variable for each production lot making the product difficult to characterize. The chemical used for inactivation, (e.g. formaldehyde), is often toxic and does not negate the possibility of reversion of the inactivated superantigen to an active form. In addition, the yields of wild-type toxin from bacterial strains used for making toxoids are often low.

SUMMARY OF THE INVENTION

The present invention relates to a vaccine which overcomes the disadvantages of the chemically inactivated toxoids described above. The superantigen vaccine(s) of the present invention is/are designed to protect individuals against the pathologies resulting from exposure to one or several related staphylococcal and streptococcal toxins. The superantigen vaccine is comprised of a purified protein product that is genetically attenuated by DNA methodologies such that superantigen attributes are absent, however the superantigen is effectively recognized by the immune system and an appropriate antibody response is produced.

Specifically, the vaccine of the present invention is a product of site-directed mutagenesis of the DNA coding sequences of superantigen toxins resulting in a disruption of binding to both the MHC class II receptor and to the T-cell antigen receptor. A comprehensive study of the relationships of the

superantigen structures of TSST-1, streptococcal pyrogenic exotoxin-A (SPEa), staphylococcal enterotoxin B (SEB), and staphylococcal enterotoxin A, to receptor binding were undertaken to provide insight into the design of the vaccine. From these studies, critical amino acid residues of the toxin responsible for binding the superantigen to the human MHC receptor were defined. Site-directed mutagenesis of the gene encoding the toxin and expression of the new protein product resulted in a superantigen toxin with disrupted binding to the MHC receptors.

Therefore, it is an object of the present invention to provide a superantigen toxin DNA fragment which has been genetically altered such that binding of the encoded altered toxin to the MHC class II or T-cell antigen receptor is disrupted. Such a DNA fragment is useful in the production of a vaccine against superantigen toxin infections.

It is another object of the present invention to provide a superantigen toxin amino acid sequence which has been altered such that the binding of the encoded altered toxin to the MHC class II or T-cell antigen receptor is disrupted. Such a sequence is useful for the production of a superantigen toxin vaccine.

It is another object of the invention to provide a recombinant vector comprising a vector and the DNA fragment described above.

It is a further object of the present invention to provide host cells transformed with the above-described recombinant DNA constructs. Host cells include cells of other prokaryotic species or eukaryotic plant or animal species, including yeasts,

fungi, plant culture, mammalian and nonmammalian cell lines, insect cells and transgenic plants or animals.

It is another object of the present invention to provide a method for producing altered superantigen toxin with disrupted MHC class II and T-cell antigen receptor binding which comprises culturing a host cell under conditions such that a recombinant vector comprising a vector and the DNA fragment described above is expressed and altered superantigen toxin is thereby produced, and isolating superantigen toxin for use as a vaccine against superantigen toxin-associated bacterial infections and as a diagnostic reagent.

It is still another object of the invention to provide a purified altered superantigen toxin useful as a vaccine and as a diagnostic agent.

It is another object of the invention to provide a purified altered superantigen toxin for the therapeutic stimulation of, or other *in vivo* manipulation of, selective T cell subsets, or *ex vivo* manipulation of T cells for *in vivo* therapeutic purposes in mammals. Diseases, such as autoimmunity, wherein T-cell responses of limited diversity (oligoclonal) are evident. Altered superantigens may be used to therapeutically inactivate (induce anergy in) T cells in diseases wherein oligoclonal T-cell responses are evident such as autoimmune diseases, for example. For diseases in which specific T-cell subsets are not active or are anergetic, altered superantigens may be used to therapeutically stimulate these T cells. Such disease include, but are not limited to, infectious diseases and cancers wherein specific subsets of cytotoxic or helper T cells are inactivated or are otherwise unable to respond normally to the antigenic stimulation of the disease moiety.

It is a further object of the present invention to provide an antibody to the above-described altered superantigen toxin for use as a therapeutic agent and as a diagnostic agent.

It is yet another object of the invention to provide a superantigen toxin vaccine comprising an altered superantigen toxin effective for the production of antigenic and immunogenic response resulting in the protection of an animal against superantigen toxin infection.

It is a further object of the invention to provide a multivalent superantigen toxin vaccine comprising altered toxins from a variety of streptococcal and staphylococcal toxins effective for the production of antigenic and immunogenic response resulting in the protection of an animal against infection with bacterial superantigen toxin-expressing strains and against other direct or indirect exposures to bacterial superantigen toxins such as might occur by ingestion, inhalation, injection, transdermal or other means.

It is yet another object of the present invention to provide a method for the diagnosis of superantigen toxin-associated bacterial infection comprising the steps of:

(i) contacting a sample from an individual suspected of having a superantigen toxin-associated bacterial infection with antibodies which recognize superantigen toxin using antibodies generated from the altered superantigen toxin; and

(ii) detecting the presence or absence of a superantigen-associated bacterial infection by detecting the presence or absence of a complex formed between superantigen toxin in said sample and antibodies specific therefor.

It is yet another object of the present invention to provide a method for the diagnosis of superantigen bacterial infection comprising the steps of:

(i) contacting a sample from an individual suspected of having the disease with lymphocytes which recognize superantigen toxin produced by said superantigen bacteria or lymphocytes which recognize altered superantigen toxin; and

(ii) detecting the presence or absence of responses of lymphocytes resulting from recognition of superantigen toxin. Responses can be, for example, measured cytokine release, increase of activation markers, mitotic activity, or cell lysis. The lymphocytes responding to the altered superantigen toxins recognize them as recall antigens not as superantigens, therefore the response is an indicator of prior exposure to the specific superantigen. The absence of a response may indicate no prior exposure, a defective immune response or in some cases a manifestation of T-cell anergy. Anergy is defined here as antigen-specific or a generalized non-responsiveness of subsets of T cells.

It is a further object of the present invention to provide a diagnostic kit comprising an antibody against an altered superantigen toxin and ancillary reagents suitable for use in detecting the presence of superantigen toxin in animal tissue or serum.

It is another object of the present invention to provide a detection method for detecting superantigen toxins or antibodies to superantigen toxin in samples, said method comprising employing a biosensor approach. Such methods are known in the art and are described for example in Karlsson *et al.* (1991) *J. Immunol. Methods* **145**, 229-240 and Jonsson *et al.* (1991) *Biotechniques* **11**, 620-627.

It is yet another object of the present invention to provide a therapeutic method for the treatment or amelioration of symptoms of superantigen-associated bacterial infection, said method comprising providing to an individual in need of such treatment an effective amount of sera from individuals immunized with one or more altered superantigen toxins from different bacteria in a pharmaceutically acceptable excipient.

It is further another object of the present invention to provide a therapeutic method for the treatment or amelioration of symptoms of superantigen toxin-associated bacterial infection, said method comprising providing to an individual in need of such treatment an effective amount of antibodies against altered superantigen toxins in a pharmaceutically acceptable excipient.

It is another object of the present invention to provide a therapeutic method for the treatment or amelioration of symptoms of bacterial superantigen toxin infection, said method comprising providing to an individual in need of such treatment an effective amount of altered superantigen from a variety of streptococcal and staphylococcal bacteria in order to inhibit adhesion of superantigen bacterial toxin to MHC class II or T-cell receptors by competitive inhibition of these interactions.

It is yet another object of the present invention to provide a therapeutic method for the treatment of diseases that may not be associated directly with superantigen toxins but which result in specific nonresponsiveness of T-cell subsets, said method comprising the administration of altered superantigen toxins, *in vivo* or *ex vivo*, such that T-cell subsets are expanded or stimulated. Diseases which cause

anergy or nonresponsiveness of T-cells include, but are not limited to, infectious diseases.

It is another object of the present invention to provide a therapeutic method for the treatment of diseases associated with expanded or over-stimulated T-cell subsets, such as autoimmunity for example, said method comprising administration of altered superantigen toxin, *in vivo* or *ex vivo*, such that anergy or inactivation of disease associated T-cells is produced. In this case, superantigen mutants can be designed with altered but not attenuated T-cell receptor binding, to cause anergy of only the select (i.e. 1-3) T-cell subsets that are pathologically activated.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1. Staphylococcal and streptococcal superantigen amino acid sequence homologies, compiled with Genetics Computers Group of Univ. of Wisconsin software.

Figure 2. Comparison of mutant SEB and SEA biological activities.

A. SEB mutant HLA-DR1-binding; **B.** SEA mutant HLA-DR1-binding; **C.** T-cell recognition of SEA and SEB mutants. Binding of bacterial superantigens to cell surface DR1 was measured by laser fluorescence-activated flow cytometry. A representative experiment of three performed is shown. The mutants SEA D197N, the homologous SEB D199N, and SEA L11Y had no effect on binding or T-cell recognition, and were used for

controls. Human T-cell proliferation, assessed by [^{3}H]thymidine incorporation, was measured in response to SEA (Y64A) or SEB (Y61A) mutants and controls that retained DR1-binding affinities. Each data point represents the mean of triplicate determinations; SEM <5%.

Figure 3. Sequence and secondary structural alignment of bacterial superantigen toxins. Analyses were performed with the applications PILEUP and PROFILE from the Computer Genetics Group (Madison, WI) using sequence data obtained from a variety of sources. Amino acid residue numbering is based on the SEA sequence.

Figure 4. Detection of TNF-α (a), IL-1α (B), IL-6 (C) and IFN-γ (D) in the serum of mice injected with SEA (open circles), LPS (open triangles), or SEA plus LPS (open squares). Values for TNF-α and IL-1α represent the mean of duplicate samples, with an SEM of ± 5%. INF-γ and IL-6 values represent the mean of duplicate and triplicate samples, respectively. The SEMs for IFN-γ and IL-6 readings were ± 5% and ± 10%, respectively.

Figure 5. Mutant SEA vaccines that have attenuated major histocompatibility complex class II or T-cell antigen receptor binding do not induce T-cell anergy. Mice were given three doses of wild type (WT) SEA or site-specific mutant vaccine, plus adjuvant. Control animals received adjuvant alone or were untreated; 2 weeks after final injection, pooled mononuclear cells were collected from spleens of 4 mice from each group. Results are represented as mean cpm (±SD) of quadruplicate wells incubated with 100 ng/ml WT SEA for 72 h and then pulse-labeled for 12 h with [^{3}H]thymidine. P<0.0001 (analysis of variance

for repeated measures comparing untreated, adjuvant, Y64A, and Y92A to WT SEA group).

Figure 6. No superantigen-induced T-cell anergy is exhibited by rhesus monkeys immunized with the vaccine B899445. Peripheral blood lymphocytes were incubated with titrated concentrations of wild-type superantigens from individual rhesus monkeys (K422 and N103) that were immunized with B899445. T-cell proliferation was assessed by [^{3}H]thymidine incorporation. Each data point represents the mean of triplicate determinations; SEM <5%.

Figure 7. Antibody responses of rhesus monkeys immunized with a combined vaccine consisting of B899445 (SEB) and A489270 (SEA). The antibody levels were measured by ELISA, using plates coated with SEA, SEB or SEC1 as listed. Monkey G8 is a non-immunized control. SEM <5% for triplicate measurements.

DETAILED DESCRIPTION

The present invention relates in part to a vaccine against superantigen toxin-associated bacterial diseases. The superantigen vaccines used in this study were developed by engineering changes in the receptor-binding portions of superantigen toxins to reduce receptor-binding affinities and toxicity while maintaining antigenicity.

Five different superantigen vaccines are described in this application: staphylococcal enterotoxin A, staplylococcal enterotoxin B, staphylococcal enterotoxin C1, toxic-shock syndrome toxin-1, and streptococcal pyrogenic exotoxin-A. For each of the superantigen toxins above, a comprehensive study of the relationships of the toxin protein structure to receptor binding was undertaken to provide insight into the design of the vaccine. The

study employed site-directed mutagenesis of toxin and receptor molecules, molecular modeling, protein structure and binding studies. Following these studies, toxins were altered by site-directed mutagenesis to attenuate MHC class II binding and biological activity to an essentially non-specific level. The engineered vaccines were evaluated at each stage of analysis to determine mouse and human T-cell reactivities *in vitro*, serological responses and toxicity in mice and monkeys.

In one embodiment, the present invention relates to an altered superantigen toxin protein having an amino acid sequence which has been altered such that the binding of the toxin to the MHC class II receptor is disrupted.

Comparison of amino acid sequences (**Fig. 1**) suggested that bacterial superantigens fall into groups consisting of (1) SEA, SED and SEE, (2) SEB, staphylococcal enterotoxins C1-C3 (SEC1-3), the streptococcal pyrogenic exotoxins A (SPE-A) and C (SPE-C), (3) TSST-1 and (4) the exfoliative toxins (ETA, ETB) and streptococcal pyrogenic exotoxin B (SPE-B), which are the most distant from the others in sequence. Although not available to the inventor when the inventions were first conceived and proof of principle was obtained, the x-ray crystallographic structures of several bacterial superantigens are now known. Diverse superantigens, such as SEB and TSST-1, appear to have little sequence in common, yet they exhibit homologous protein folds composed largely of β strands [Prasad, G.S. *et al.* (1993) *Biochemistry* **32**, 13761-13766; Acharya, R.K. *et al.* (1994) *Nature* **367**, 94-97; Swaminathan, S. *et al.* (1992) *Nature* **359**, 801-806] within two distinct domains. Differences between the proteins are located primarily in highly variable

regions comprised of several surface loops, such as the disulfide-bonded loop which is absent from TSST-1 and at the amino terminus.

The X-ray crystal structures of SEB and TSST-1 complexed with HLA DR1 are known [Kim, J. *et al.* (1994) *Science* **266**, 1870-1874 ; Jardetzky, T.S. *et al.* (1994) *Nature* **368**, 711-718]. The region of HLA DR1 that contacts SEB consists exclusively of α subunit surfaces. The main regions of SEB involved are two conserved sites: a polar pocket derived from three β strands of the β barrel domain and a highly solvent-exposed hydrophobic reverse turn. The polar binding pocket of SEB contains a glutamate and two tyrosines that accommodate Lys39 of the α subunit of HLA DR1, while the hydrophobic region consists of a leucine and flanking residues that make several contacts with the HLA DRα chain. The HLA DR1 binding sites of both TSST-1 and SEB overlap significantly. The hydrophobic binding contacts of other SAg with the HLA DRα chain have been proposed [Ulrich, *et al.* (1995). *Nature, Struct. Biol* **2**, 554-560] to be similar to those found in SEB and TSST-1. A motif consisting of a leucine in a reverse turn [Ulrich *et al.* (1995), *supra*] is conserved among bacterial superantigens and may provide the key determinant (hydrophobic or otherwise) for binding HLA-DR. However, TSST-1 does not have a highly charged residue in the polar pocket that interacts with Lys39 of the HLA DRα chain and uses an alternative conformational binding mode that allows TSST-1 to interact with HLA DR1 β-chain residues and the carboxy-terminal region of the antigenic peptide.

Both SEA and SEE bind to the β subunit of DR by means of a single zinc atom [*Fraser*, J.D. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5507-5511]. The amino-terminal domain of SEA interfaces with the HLA

DRα chain [Ulrich, *et al.* (1995)], while SEA C-terminal domain residues His187, His225 and Asp227 form a zinc-coordination complex, likely with His-81 from the β chain of an adjoining HLA DR molecule. However, our results have shown that binding of superantigen to the HLA DRβ subunit does not directly stimulate T cells [Ulrich *et al.* (1995) *Nature, Struct. Biol.* 2, 554-560], but increases the potential of the bound SEA to interact with the α chain of another HLA DR, thus increasing the biological potency.

A least-squares superimposition of the unbound molecules of modeled SEA and the crystal structure of SEB, aligned according to their structurally conserved α-helical and ß-strand regions, exhibited a global folding pattern which is very similar. Differences between the two structures are calculated to be located primarily in loops of low sequence homologies, with the largest positional deviations occurring between structurally conserved regions of residues 18-20, 30-32, 173-181, 191-194, and the cysteine-loop region (90-111). Only one of these regions in SEB makes significant contact (residue Y94 [Y=tyrosine] in particular) with the HLA-DR1 molecule [Jardetzky, T.S. *et al.* (1994) *Nature* **368**, 711-718].

The binding interface between SEB and HLA-DR1 consists principally of two structurally conserved surfaces located in the N-terminal domain: a polar binding pocket derived from three ß-strand elements of the ß-barrel domain and a hydrophobic reverse turn. The binding pocket of SEB contains residues E67 (E=Glutamic acid), Y89 (Y=Tyrosine) and Y115 (Y=tyrosine), and binds K39 (K=Lysine) of the DRα subunit. The amino acid one letter code is defined as the following: A= Alanine (Ala), I= Isoleucine (Ile),

L= Leucine (Leu), M= Methionine (Met), F= Phenylalanine (Phe), P= Proline (Pro), W=Tryptophan (Trp), V=Valine (Val), N= Asparagine (Asn), C=Cysteine (Cys), Q= Glutamine (Q), G= Glycine (Gly), S= Serine (Ser), T= Threonine (Thr), Y= Tyrosine (Tyr), R= Arginine (Arg), H=Histidine (His), K= Lysine (Lys), D= Aspartic acid (Asp), and E= Glutamic acid (Glu). For SEA, the binding interface with the DR molecule is modeled to contain a similar binding pocket consisting of residues D70, Y92 and Y108. Mutation of residue Y89 in SEB or Y92 in SEA to alanine (**Fig. 2**) resulted in greater than 100-fold reduction in DR1 binding. The substitution of alanine for Y89 in SEB and Y92 in SEA eliminates the hydrogen bond with K39 and disrupts packing interactions with adjacent protein residues. Modeling of the SEA mutant Y92A predicts an increase in solvent-accessible surface area for Y108 by a factor of two greater than the wild-type structure, allowing the formation of a hydrogen bond to the carboxylate group of D70 and thus disrupting key anchoring and recognition points for HLA-DR1. This effect is expected to be somewhat less in SEB due to the longer side chain at E67. Substitution of SEB Y115 with alanine also resulted in greater than 100-fold reduction of binding. In contrast, the same replacement of Y108 in SEA yielded little to no change in DR1 binding (**Fig. 2a**), suggesting the primary importance of SEA residues Y92 and D70 for stabilizing interactions with K39. The K39 side chain of DRα forms a strong ion-pair interaction with the SEB E67 carboxylate group and hydrogen bonds with the hydroxyl groups of Y89 and Y115. Substitution of SEB E67 by glutamine reduced binding affinity by greater than 100-fold (**Fig. 2**), reflecting the replacement of the strong ionic bond with a weaker hydrogen bond. To

optimize ion-pair interactions of the analogous SEA site, the shorter carboxylate side chain of D70 is predicted to shift K39 of DRα, weakening interactions with SEA Y108. The substitution of alanine for SEA Y108 is thus more easily accommodated than the homologous substitution of SEB Y115, without loss in DR1 binding.

Comparisons of the polar pocket with other bacterial superantigens were then made. SEC1-3 and SPE-A have conserved the critical DR1 binding-interface residues (**Fig. 1**), and share with SEB and SEA secondary structural elements of the DR1-binding surfaces. Asparagine in SED (N70) replaces the acidic side chain present in SEA, SEB, SPE-A and SEC1-3. Accordingly, for SED the salt bridge of the polar pocket is likely to be replaced by a hydrogen bond. Overall, DR1 affinities for SED and SEA appeared to be equivalent (**Fig. 2b**), indicating that other interactions may compensate for the absence in SED of the ion-pair found in the other superantigens. For the case of TSST-1, mutating DRα residues K39 to serine or M36 to isoleucine has been shown to greatly reduce binding [Panina-Bordignon *et al.* (1992) *J. Exp. Med.* **176**: 1779-1784]. Although primarily hydrophobic, the critical TSST-1 structural elements are conserved with the SEA and SEB polar binding pocket. SEB residues Y89 and Y115 are homologous to T69 and I85 in TSST-1, respectively, and SEB E67 is replaced by I46. These TSST-1 residues are positioned in a conserved ß-barrel domain found in both SEB and SEA. However, the TSST-1 site lacks polarity equivalent to SEB/SEA, and hydrogen bonding with the hydroxyl of TSST-1 residue T69 would require that DRα K39 extend 5 Å into the pocket. TSST-1 binding utilizes an alternative strategy [Kim *et al.* (1994)

Science **266**:1870-1874] consisting of hydrophobic contacts centered around residue I46, and potential ionic or hydrogen bonds bridging DRα residues E71 and K67 to R34 and D27, respectively, of TSST-1.

The hydrophobic region of the binding interface between SEB and the HLA-DR1 molecule consists of SEB residues 44-47, located in a large reverse turn connecting ß-strands 1 and 2 of SEB. These residues appear to make strong electrostatic interactions with DRα through their backbone atoms. The mutation of L45 to an arginine reduced overall HLA-DR1 binding greater than 100-fold (**Fig. 2b**), attributable to the less energetically favorable insertion of a highly charged residue into a hydrophobic depression on the DR1 molecule. The modeled DR1-SEA complex presents similar interactions with the SEA backbone atoms, with the exception of a glutamine (Q49) replacing SEB Y46. Mutation of L48 to glycine in SEA (homologous to L45 of SEB) has been reported to decrease T-cell responses. SEB L45 and the comparable L30 of TSST-1 are the most extensively buried residues in the DR1 interface. The leucine is conserved among the bacterial superantigens (**Fig. 3**) and may provide the necessary hydrophobic structural element for surface complementarity with DR1, consistent with the mutagenesis data for SEB and SEA.

The inventor has performed similar structure and function studies with TSST-1, SEC1 and SPE-A.

In determining the overall affinity of the superantigen for DR1, a contributory role is played by structural variations around the common binding motifs. A short, variable structured, disulfide-bonded loop is found in SEA and a homologous longer loop in SEB. The SEB residue Y94, contained within this loop, forms hydrophobic interactions with L60 and

A61 of the DRα subunit. Replacement of Y94 with alanine partially inhibits DR1 binding (**Fig. 2a,b**). An alanine is found in SEA (A97) and SEE at the position equivalent to SEB Y94, and mutating this residue in SEA to tyrosine results in disrupted instead of stabilized interactions with DR1 (**Fig. 2a**). Although the disulfide loops differ in structure between SEA and SEB, A97 apparently contributes to the DRα binding interface in a manner similar to Y94 of SEB. Because TSST-1 lacks a disulfide loop, similar contacts with DRα are replaced by interactions with β-strands of TSST-1. In a like manner, the absence of a salt bridge between the residues K39 of DRα and N65 of SED is apparently compensated for by stabilizing interactions occurring outside of the otherwise conserved dominant binding surfaces (**Fig. 2a**).

The amino acid residues in contact with TCR are located in regions of high sequence variability, presenting a unique surface for interaction with the TCR. Residues implicated in TCR interactions by mutagenesis of SEA and SEB reside in variable loop regions, while TSST-1 mutants that affect TCR binding are mainly located in an α helix [Acharya, R.K. *et al.* (1994) *Nature* **367**, 94-97; Kim, J. *et al.* (1994) *Science* **266**, 1870-1874]. Specifically, mutations that diminish T-cell receptor recognition of SEB include residues N23, Y61, and the homologous SEA N25 or Y64 **(Fig. 2c)**. SEA residues S206 and N207 also control T-cell responses [Hudson, *et al.*(1992) *J. Exp. Med.* **177**: 175-184]. Mutants of the polar binding pocket, SEA Y92A and SEB Y89A, equivalently reduced T-cell responses (**Fig. 2c**), reflecting the observed decreases in DR1-binding (**Fig. 2a, b**). While supporting reduced T-cell responses, mutants SEA Y64A

and SEB Y61A retained normal affinities for DR1 (**Fig. 2a-c**).

In view of the detailed description of the present invention and the results of molecular modelling and structural studies of staphylococcal and streptococcal superantigen toxins discussed above, any amino acid sequence derived from a superantigen toxin can be altered. Sequences of several superantigen toxins are already known and available to the public in sequence databases such as GenBank, for example. The superantigen toxin sequence is preferably altered at the hydrophobic loop or polar binding pocket depending on the superantigen. Alternatively, residues adjacent to the hydrophobic loop or polar binding pocket that contact HLA-DR or residues at sites that can indirectly alter the structure of the hydrophobic loop or polar pocket can be altered. The number of residues which can be altered can vary, preferably the number can be 1-2, more preferably 2-3, and most preferably 3-4, or more with the limitation being the ability to analyze by computational methods the consequences of introducing such mutations. The residues which can be altered can be within 5 amino acid residues of the central Leucine of the hydrophobic loop (such as L45 of SEB), or within 5 residues of one of the amino acid residues of the polar binding pocket that can contact HLA-DR, (such as E67, Y89, or Y115 of SEB), more preferably, within 3 amino acid residues of the central Leucine of the hydrophobic loop (such as L45 of SEB), or within 3 residues of one of the amino acid residues of the polar pocket that can contact HLA-DR, (such as E67, Y89, or Y115 of SEB), and most preferably, the central Leucine of the hydrophobic loop (such as L45 of SEB), or one of the amino acid residues of the polar binding

pocket that can contact HLA-DR, (such as E67, Y89, or Y115 of SEB). The residues can be changed or substituted to alanine for minimal disruption of protein structure, more preferably to a residue of opposite chemical characterisitcs, such as hydrophobic to hydrophilic, acidic to neutral amide, most preferably by introduction of a residue with a large hydrated side chain such as Arginine or Lysine. In addition, side chains of certain nonconserved receptor-binding surfaces, can also be altered when designing superantigen toxins with low binding affinities. These residues can include Y94 of SEB and structurally equivalent residues of other superantigens, such as A97 of SEA, or any side chain within 5 residues from these positions or any side chain in discontinuous positions (discontinuous positions are defined as amino acid residues that fold together to form part of a discrete three-dimensional structural unit but are not present on the same secondary structural unit e.g. α helix or β-strand) such as disulfide-bonded side chains, that involve, directly or indirectly, the nonconserved receptor contact surfaces outside of the polar binding pocket or hydrophobic loop. Further, amino acid residues involved with protein folding or packing can be altered when designing superantigen toxins with low binding affinities [Sundstrom *et al.* (1996) *EMBO J.* **15**, 6832-6840; Sundstrom *et al.* (1996) *J. Biol. Chem.* **271**, 32212-32216; Acharya *et al.* (1994) *Nature* **367**, 94-97; Prasad *et al.* (1993) *Biochem.* **32**, 13761-13766; Swaminathan *et al.* (1992) *Nature* **359**, 801-806]. Furthermore, especially for superantigens with higher affinities for T-cell antigen receptors, side chains of amino acids within 5 residues of the position represented by N23 (conserved residue in most

superantigens) , N60 (conserved Asn or Trp in most superantigens) Y91 (semiconserved hydrophobic residues Trp, Ile, Val, His in most superantigens) and D210 of SEB (conserved Asp in most superantigens) can be altered when designing superantigen toxins with low binding affinities. These residues are likely to form part of the integral molecular surfaces that are in contact with T-cell antigen receptors. Because the T-cell receptor contact areas of superantigen toxins are essential for causing specific activation or inactivation of T-cell subsets, altering residues that are unique to each superantigen but that are located within 5 residues of the positions represented by N23, N60 and Y91 can produce superantigens that affect a smaller number (e.g. 1-3) of subsets. Such altered superantigen toxins can be useful as therapeutic agents.

In another embodiment, the present invention relates to a DNA or cDNA segment which encodes a superantigen toxin such as SEA, SEB, SEC-1, SPEa, and TSST-1 to name a few, the sequence of which has been altered as described above to produce a toxin protein with altered binding ability to MHC Class II and/or T-cell receptors. For SEA, the following three mutations were introduced into the toxin molecule: Tyrosine at amino acid position 92 changed to alanine; Aspartic acid at amino acid position 70 changed to arginine; Leucine at amino acid position 48 changed to arginine. The reduction in binding to HLA DR is additive per mutation, though one or two mutations can produce a vaccine and a combination of all three mutations in one molecule produces a better vaccine. Other substitutions can also result in reduced binding.

The B899445 vaccine consists of the following three mutations simultaneously introduced into the toxin molecule: tyrosine at amino acid position 89 changed to alanine; tyrosine at amino acid position 94 changed to alanine; leucine at amino acid position 45 changed to arginine. The altered superantigen toxins can be expressed either as a full-length propolypeptide or as a polypeptide in which the leader peptide has been deleted. The full-length expressed product (SEA vaccine, A489270P; SEB vaccine B899445P, B2360210P) is secreted into the periplasmic space of *E. coli* host cells, and the leader peptide is recognized and cleaved by a native bacterial enzymatic mechanism. The altered superantigen toxins in which the leader peptide has been deleted (A489270C, B899445C), the first residue of the mature protein is encoded by the transcriptional start site and codon for methionine (ATG), and the protein is expressed as a nonsecreted product within the host *E. coli* cell. For the TSST-1 vaccine TST30, the leucine at position 30 was changed to arginine. For the SEC1 vaccine, SEC45, the leucine at position 45 was changed to arginine. For the SPE-A vaccine, SPEA42, the leucine at position 42 was changed to arginine.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The vector can take the form of a plasmid such as any broad host range expression vector for example pUC18/19, pSE380, pHIL, pET21/24 and others known in the art. The DNA sequence is preferably functionally linked to a promoter such that the gene is expressed when present in an expression system and an altered superantigen toxin is produced. The expression system can be an *in vitro* expression system or host cells

such as prokaryotic cells, or *in vivo* such as DNA vaccines.

In a further embodiment, the present invention relates to host cells stably or transiently transformed or transfected with the above-described recombinant DNA constructs. The host can be any eukaryotic or prokaryotic cell including but not limited in *E. coli* DH5α or BL21. The vector containing the altered superantigen toxin gene is expressed in the host cell and the product of the altered toxin gene, whether a secreted mature protein or a cytoplasmic product, can be used as a vaccine or as a reagent in diagnostic assays or detection methods, or for therapeutic purposes. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a highly purified IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of altered toxin. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the altered toxin described above.

A recombinant or derived altered superantigen toxin is not necessarily translated from a designated nucleic acid sequence; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system. In addition the altered toxin can be fused to other proteins or polypeptides for directing transport for example into the periplasm or for secretion from the

cell. This includes fusion of the recombinant or derived altered superantigen to other vaccines or sequences designed to aid in purification, such as His-tagged, epitope-tagged or antibody Fc-fusions.

In a further embodiment, the present invention relates to a method of producing altered superantigen toxin which includes culturing the above-described host cells, under conditions such that the DNA fragment is expressed and a superantigen toxin protein is produced. The superantigen toxin can then be isolated and purified using methodology well known in the art such as immunoaffinity chromatography or preparative isoelectric focusing. However, the method of purification is not critical to the performance of the vaccine. The altered superantigen toxin can be used as a vaccine for immunity against infection with bacterial superantigen toxins or as a diagnostic tool for detection of superantigen toxin-associated disease or bacterial infection. The transformed host cells can be used to analyze the effectiveness of drugs and agents which affect the binding of superantigens to MHC class II or T-cell antigen receptors. Chemically derived agents, host proteins or other proteins which result in the down-regulation or alteration of expression of superantigen toxins or affect the binding affinity of superantigen toxins to their receptors can be detected and analyzed. A method for testing the effectiveness of a drug or agent capable of altering the binding of superantigen toxins to their receptors can be for example computer-aided rational design or combinatorial library screening, such as phage display technology.

In another embodiment, the present invention relates to antibodies specific for the above-described altered superantigen toxins. For instance, an

antibody can be raised against the complete toxin or against a portion thereof. Persons with ordinary skill in the art using standard methodology can raise monoclonal and polyclonal antibodies to the altered superantigens of the present invention, or a unique portion of the altered superantigen. Materials and methods for producing antibodies are well known in the art (see for example Goding, in, <u>Monoclonal Antibodies: Principles and Practice</u>, Chapter 4, 1986). The antibodies can be used in diagnostic assays for detection of superantigen toxin-associated infection. Neutralizing antibodies can be used in a therapeutic composition for the treatment of amelioration of anergy and/or for the treatment of a superantigen toxin-associated infection.

In a further embodiment, the present invention relates to a method for detecting the presence of superantigen-associated bacterial infections in a sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g. nitrocellulose membrane), all or a unique portion of the altered superantigen described above, and contacting it with the serum of a person suspected of having a superantigen-associated bacterial infection. The presence of a resulting complex formed between the altered superantigen toxin and antibodies specific therefor in the serum can be detected by any of the known methods common in the art, such as fluorescent antibody spectroscopy or colorimetry. This method of detection can be used, for example, for the diagnosis of superantigen-associated bacterial infections.

In yet another embodiment, the present invention relates to a method for detecting the presence of superantigen toxin in a sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g. nitrocellulose membrane), antibodies specific for altered superantigen toxin, and contacting it with serum or tissue sample of a person suspected of having superantigen-associated bacterial infection. The presence of a resulting complex formed between toxin in the serum and antibodies specific therefor can be detected by any of the known methods common in the art, such as fluorescent antibody spectroscopy or colorimetry. This method of detection can be used, for example, for the diagnosis of superantigen-associated bacterial infection or disease such as food poisoning and toxic-shock syndrome or the detection of superantigen toxin in food and drink.

In another embodiment, the present invention relates to a diagnostic kit which contains altered superantigen toxin from a specific bacteria or several different superantigen toxins from bacteria and ancillary reagents that are well known in the art and that are suitable for use in detecting the presence of antibodies to superantigen toxin-associated bacteria in serum or a tissue sample. Tissue samples contemplated can be avian, fish, or mammal including monkey and human.

In yet another embodiment, the present invention relates to a vaccine for protection against superantigen toxin-associated bacterial infections. The vaccine can comprise one or a mixture of individual altered superantigen toxins, or a portion thereof. When a mixture of two or more different

altered superantigen toxin from different bacteria is used, the vaccine is referred to as a multivalent bacterial superantigen vaccine. The vaccine is designed to protect against the pathologies resulting from exposure to one or several related staphylococcal and streptococcal toxins. In addition, the protein or polypeptide can be fused or absorbed to other proteins or polypeptides which increase its antigenicity, thereby producing higher titers of neutralizing antibody when used as a vaccine. Examples of such proteins or polypeptides include any adjuvants or carriers safe for human use, such as aluminum hydroxide.

The staphylococcal enterotoxin (SE) serotypes SEA, SED, and SEE are closely related by amino acid sequence, while SEB, SEC1, SEC2, SEC3, and the streptococcal pyrogenic exotoxins B share key amino acid residues with the other toxins, but exhibit only weak sequence homology overall. However, there are considerable similarities in the known three-dimensional structures of SEA, SEB, SEC1, SEC3, and TSST-1. Because of this structural similarity, it is likely that polyclonal antibodies obtained from mice immunized with each SE or TSST-1 exhibit a low to high degree of cross-reaction. In the mouse, these antibody cross-reactions are sufficient to neutralize the toxicity of most other SE/TSST-1, depending upon the challenge dose. For example, immunization with a mixture of SEA, SEB, TSST-1 and SPEa was sufficient to provide antibody protection from a challenge with any of the component toxins, singly or in combination.

The likelihood of substantial antigen-cross-reactivity suggests that it may be possible to obtain immune protection for other (or perhaps all) staphylococcal superantigens by use of a minimal mixed

composition of vaccines. For the case of staphylococcal superantigens, a combination of the component vaccines from SEA, SEB, SEC-1 and TSST-1 should be sufficient to provide immune protection against SEA, SEB, SEC1-3, and TSST-1. The addition of SPEa component to the trivalent mixture will allow for sufficient protection against the streptococcal toxins SPEa and SPEc. Therefore, a multivalent vaccine consisting of the altered superantigen toxins from SEA, SEB, SEC-1, TSST-1, and SPEa as described above, is predicted to provide protective immunity against the majority of bacterial superantigen toxins.

The vaccine can be prepared by inducing expression of a recombinant expression vector comprising the gene for the altered toxin described above. The purified solution is prepared for administration to mammals by methods known in the art, which can include filtering to sterilize the solution, diluting the solution, adding an adjuvant and stabilizing the solution. The vaccine can be lyophilized to produce a vaccine against superantigen toxin-associated bacteria in a dried form for ease in transportation and storage. Further, the vaccine may be prepared in the form of a mixed vaccine which contains the altered superantigen toxin(s) described above and at least one other antigen as long as the added antigen does not interfere with the effectiveness of the vaccine and the side effects and adverse reactions, if any, are not increased additively or synergistically. Furthermore, the vaccine may be administered by a bacterial delivery system and displayed by a recombinant host cell such as *Salmonella* spp, *Shigella* spp, *Streptococcus* spp. Methods for introducing recombinant vectors into host cells and introducing host cells as a DNA delivery

system are known in the art [Harokopakis *et al.* (1997) *Infect. Immun.* **65**, 1445-1454; Anderson *et al.* (1996) *Vaccine* **14**, 1384-1390; Medaglini *et al.* (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6868-6872].

The vaccine may be stored in a sealed vial, ampule or the like. The present vaccine can generally be administered in the form of a liquid or suspension. In the case where the vaccine is in a dried form, the vaccine is dissolved or suspended in sterilized distilled water before administration. Generally, the vaccine may be administered orally, subcutaneously, intradermally or intramuscularly but preferably intranasally in a dose effective for the production of neutralizing antibody and protection from infection or disease.

In another embodiment, the present invention relates to a method of reducing superantigen-associated bacterial infection symptoms in a patient by administering to said patient an effective amount of anti-altered superantigen toxin antibodies, as described above. When providing a patient with anti-superantigen toxin antibodies, or agents capable of inhibiting superantigen function to a recipient patient, the dosage of administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of the above compounds which is in the range of from about 1pg/kg to 10 mg/kg (body weight of patient), although a lower or higher dosage may be administered.

In a further embodiment, the present invention relates to a therapeutic method for the treatment of diseases that may not be associated directly with superantigen toxins but which result in specific

nonresponsiveness of T-cell subsets or detection of abnormally low level of subsets in peripheral blood, said method comprising the administration of altered superantigen toxins, *in vivo* or *ex vivo*, such that T-cell subsets are expanded or stimulated. Diseases which cause anergy or nonresponsiveness of T-cells include, but are not limited to, infectious diseases and cancers. The desired clinical outcome such as an increase in detectable T cell subsets or in stimulation *ex vivo* of T-cells through their antigen receptors, such as by antigen or anti-CD3 antibody can be measured by standard clinical immunology laboratory assays.

In yet another embodiment, the present invention relates to a therapeutic method for the treatment of diseases associated with expanded or over-stimulated T-cell subsets, such as autoimmunity for example, said method comprising administration *in vivo* or *ex vivo*, of superantigen toxin altered in such a manner that only limited (1-3) T-cell subsets are stimulated but that MHC class II binding affinity still remains, such that anergy or inactivation of T-cells is produced. The desired clinical outcome can be measured as a reduction of circulating blood T-cells of the targeted subset(s) or diminished antigen or other antigen receptor-mediated-stimulatory responses by assays known in the art.

Described below are examples of the present invention which are provided only for illustrative purposes, and not to limit the scope of the present invention. In light of the present disclosure, numerous embodiments within the scopy of the claims will be apparent to those of ordinary skill in the art.

The following Materials and Methods were used in the Examples that follow.

Structural comparisons

Primary protein structure data are available for several bacterial superantigens, including SEA, SED, SEB, SEC1-3, TSST-1. Superantigens for which structures were unavailable were modeled using comparative techniques (HOMOLOGY program; Biosym Technologies, Inc., San Diego, CA). Before x-ray crystallography data was available, SEA was modeled by using this method, and the model was in very close agreement with the experimentally determined structure. As an example, the amino acid sequence for SEA was aligned with the known structure of free and HLA-DR1 bound SEB, and the SEA molecule was built for both free and DR1-bound proteins. Loop segments of SEA were generated by a *de novo* method. Refinement of the modeled structures was carried out by means of molecular-dynamics simulations (DISCOVER, Biosym). The constructed free SEA molecule was immersed in a 5-Å layer of solvent water and the α-carbon atoms lying in the structurally conserved regions were tethered to their initial positions during the simulations. For the bound SEA molecule, simulations were carried out by constructing an active-site region composed of part of the SEA molecule and the DR1 molecule inside a 10-Å interface boundary, as derived from the crystal structure of the DR1-SEB complex. Amino acid residues lying in the outer boundary were rigidly restrained at their initial positions. The active-site region was immersed in a 5-Å layer of water. Protein interactions were modeled by employing the consistent valence force field with a non-bonded cutoff distance of 11.0 Å. Simulations were initiated with 100 cycles of minimization using a steepest descent algorithm

followed by 100-ps relaxation (using a 1.0 fs timestep). Structural comparisons between SEB, SEC1, and TSST-1 were performed by using the crystal structures (Brookhaven data holdings) aligned according to common secondary structural elements and/or by sequence and structural homology modeling.

Site-specific mutagenesis

Site-specific mutagenesis was performed according to the method developed by Kunkel, using gene templates isolated from Staphylococcus aureus strains expressing SEA (FDA196E, a clinical isolate, Fraser, J.D. (1994) *Nature* **368**: 711-718), SEB (14458, clinical isolate), SEC1 (Toxin Technologies, Sarasota, FL), TSST-1 (pRN6550 cloned product, a clinical isolate, Kreiswirth, B. N. *et al.* (1987) *Mol. Gen. Genet.* **208**, 84-87), and SPEa (Toxin Technologies), respectively. Modified T7 polymerase (Sequenase, U.S. Biochemical Corp., Cleveland, OH) was used to synthesize second-strand DNA from synthetic oligonucleotides harboring the altered codon and single-stranded, uracil-enriched M13 templates. Mutagenized DNA was selected by transforming *E. coli* strain JM101. Alternatively, double stranded DNA was used as template for mutagenesis. Mutagenized sequences were confirmed by DNA sequencing (Sanger *et al.*, 1977, *Proc. Natl. Acad. Sci. USA* **74**: 5463-5467; Sambrook *et al.*, 1989) using synthetic primers derived from known sequences, or universal primers. The complete coding sequences were inserted into expression plasmids such as pUC19, pSE380 or pET21 for production in *E. coli* hosts.

Protein purifications

The appropriate *E. coli* hosts were transformed with plasmids harboring the mutant toxin genes. In general, the bacteria were grown to an A600 0.5-0.6 in Terrific Broth (Difco Laboratories, Detroit, MI) containing 50 μg/mL ampicillin or kanamycin. Recombinant proteins were induced with isopropyl-ß-D-thio-galactopyranoside (Life Technologies, Gaithersburg, MD) and recovered as cytoplasmic or bacterial periplasmic secretion products. Bacteria were collected by centrifugation, washed with 30 mM NaCl, 10 mM TRIS (pH 7.6), and pelleted by centrifugation and either lysed or osmotically shocked for collection of secreted proteins. Preparations were isolated by CM Sepharose ion-exchange chromatography, rabbit antibody (Toxin Technologies, Sarasota, FL) affinity columns, ion exchange HPLC or similar methods. In some cases partially purified superantigen was further purified by preparative isoelectric focusing (MinipHor; Rainin Instrument Company, Inc., Woburn, MA.). The MinipHor was loaded with the SEA-enriched fraction from CM Sepharose chromatography in a solution containing 10% (v/v) glycerol and 1% (v/v) pH 6-8 ampholytes (Protein Technologies, Inc., Tucson, AZ). The protein preparations were allowed to focus until equilibrium was reached (approximately 4 hr, 4°C). Twenty focused fractions were collected and aliquots of each were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The SEA-containing fractions were pooled, and refocused for an additional 4 h. The fractions containing purified SEA were pooled and dialyzed first against 1 M NaCl (48 h, 4°C) to remove ampholytes, and then against PBS (12 h, 4°C). Legitimate amino-terminal residues were

confirmed by protein sequencing. Precise measurements of protein concentrations were performed by immunoassay using rabbit antibody affinity-purified with the wild-type superantigens and by the bicinchoninic acid method (Pierce, Rockford, IL) using wild-type protein as standards. All protein preparations were >99% pure, as judged by SDS-PAGE and Western immunoblots. In some cases, as when used for lymphocyte assays, bacterial pyrogens were removed by passing the protein preparations over Polymyxin B affinity columns.

<u>Binding of superantigens to HLA-DR1</u>

The DR1 homozygous, human B-lymphoblastoid cell line LG2 or L cells transfected with plasmids encoding HLA-DR1αβ were used in the binding experiments. Cells were incubated 40 min (37°C) with wild-type or mutant superantigen in Hanks balanced salt solution (HBSS) containing 0.5% bovine serum albumin. The cells were washed with HBSS and then incubated with 5 μg of specific rabbit antibody (Toxin Technology, Sarasota, FL) for 1 h on ice. Unbound antibody was removed, and the cells were incubated with FITC-labelled goat anti-rabbit IgG (Organon Teknika Corp., Durham, N.C.) on ice for 30 min. The cells were washed and analyzed by flow cytometry (FACScan; Becton Dikinson & Co., Mountain View, CA). Controls consisted of cells incubated with affinity purified anti-toxin and the FITC labelled antibody without prior addition of superantigen.

<u>Lymphocyte proliferation</u>

Human peripheral blood mononuclear cells were purified by Ficoll-hypaque (Sigma, St. Louis, MO)

buoyant density gradient centrifugation. Genes encoding the human MHC class II molecules DR1αβ (DRA and DRB1*0101 cDNA [Bavari and Ulrich (1995) *Infect. Immun.* **63**, 423-429] were cloned into the eukaryotic expression vector pRC/RSV (Invitrogen, Carlsbad, CA), and mouse L cells were stably transfected. The transfectants were selected by fluorescence-activated cell sorting (EPICS C, Coulter Corp., Hialeah, FL) using rabbit anti-DRαβ antisera and FITC-goat anti-rabbit IgG, to produce cells that expressed a high level of DRαβ21. 1 x 10^5 cells/well of a 96-well plate were irradiated (15,000 Rad), and wild-type or mutant SE, was added. After a brief incubation period (45 min, 37°C), unbound SE was rinsed from the culture plates using warm media. The cells were cultured in RPMI-1640 (USAMRIID) with 5% FBS for 72 h, and pulsed-labelled for 12 h with 1μCi [^{3}H]-thymidine (Amersham, Arlington Heights, IL). Cells were harvested onto glass fiber filters, and [^{3}H]-thymidine incorporation into the cellular DNA was measured by a liquid scintillation counter (BetaPlate, Wallac Inc., Gaithersburg, MD). Splenic mononuclear cells or human peripheral blood mononuclear cells were obtained by buoyant density centrifugation (Histopaque; Sigma Chemical Comp.) and washed three times. The cells were resuspended in medium containing 5% fetal bovine serum (FBS), and 100 μl (4 x 10^5 cells) of the cell suspension was added to triplicate wells of 96-well flat bottom plates. The mononuclear cells were cultured (37°C, 5% CO_2) with WT or mutant SEA. After 3 days the cultures were pulsed (12h) with 1 μCi/well of [^{3}H]thymidine (Amersham, Arlington Heights, IL) and incorporated radioactivity was measured by liquid scintillation.

Gel electrophoresis and immunoblotting analysis.

The protein preparations were analyzed by SDS-PAGE (12%) and stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Comp. St Louis, MO) in methanol (10% v/v) acetic acid (10% v/v). The proteins separated by SDS-PAGE (not stained) were transferred to nitrocellulose membranes (Bio-Rad Lab. Inc., Melville, NY) by electroblotting, and the membranes were then blocked (12 h, 4°C) with 0.2% casein in a buffer consisting of 50 mM sodium phosphate, 140 mM sodium chloride, pH 7.4 (PBS). The membrane was then incubated (1 h, 37°C, shaking) with 2 μg/mL of affinity-purified anti-toxin antibody (Toxin Technology, Sarasota, FL) in PBS with 0.02% casein. After the membranes were thoroughly washed, peroxidase-conjugated goat anti-rabbit IgG (Cappel/Organon Teknika Corp., West Chester, PA) was added (1:5,000) and the membranes were incubated for 1 h (37°C) with shaking. The unbound antibody was removed by washing with PBS and bound antibody was visualized by using a Bio-Rad peroxidase development kit (Biorad, Hercules, CA). For quantitation, dilutions of wild-type preparations were immobilized on nitrocellulose membranes by using a Slot-Blot apparatus (Bio-Rad). The membrane was removed from the Slot-Blot apparatus and unreacted sites were blocked (12h, 4°C) with 0.2% casein in PBS. After washing once with the PBS, the membrane was incubated (1h, 37°C) with 2 μg/mL rabbit affinity purified anti-toxin antibody (Toxin Technology) in PBS that contained 0.02% casein. After four washes, the bound rabbit antibody was reacted with goat anti-rabbit IgG conjugated with horseradish peroxidase (1 h, 37°C) and the blots were developed using enhanced

chemiluminescence (ECL; Amersham Life Sciences, Arlington Heights, IL) or similar methods. The amount of mutant protein was measured by densitometry (NIH Image 1.57 software, National Institutes of Health, Bethesda, MD) of exposed X-ray film. Standard curves were prepared by plotting the mean of duplicate densitometric readings for each dilution of toxin standard. The resulting values were fitted to a straight line by linear regression. Concentrations of proteins were determined by comparing mean values of various dilutions of the mutant to the standard curve.

Biological activities and Immunizations.

Male C57BL/6 mice, 10 to 12-weeks old, were obtained from Harlan Sprague-Dawley, Inc. (Frederick Cancer Research and Development Center, Frederick, MD). The lethal effect of WT or mutant SEA was evaluated as described in Stiles *et al.* (1993) *Infect. Immun.* **61**, 5333-5338. For immunizations, mice were given by interperitoneal (ip) injections either 2 or 10 μg of WT or mutant toxin in 100 μl of adjuvant (RIBI, Immunochem Research, Inc. Hamilton, MT or alum), or adjuvant only, and boosted (ip) at 2 and 4 weeks. Serum was collected from tail veins one week after the last immunization. Mice were challenged 2 weeks after the last injection with toxin and lipopolysaccharide (LPS, 150 μg) from *E. coli* 055:B5 serotype (Difco Laboratories, Detroit, MI). Challenge controls were adjuvant-immunized or non-immunized mice injected with both agents (100% lethality) or with either wild type toxin or LPS. No lethality was produced by these negative controls. Monkeys were immunized with the antigen in the right leg, caudal thigh muscles. Each received three intramuscular immunizations with a superantigen vaccine plus

adjuvant. Control monkeys received 0.5 ml total volume of adjuvant (Alhydrogel, Michigan Department of Public Health) and sterile PBS using the same techniques and equipment as the immunized monkeys. Immunizations were administered 28±2 days apart and consisted of 20 μg of the vaccine in adjuvant in a total volume of 0.5 ml. Immunizations were administered on day 0, 28±2, and 56±2 using a 23-27 ga 1/2-5/8" needle attached to a 1 ml tuberculin syringe into the caudal thigh.

Antibody assay.

Microtiter plates were coated with 1 μg/well of WT toxin in 100 μl of PBS (37°C, 2 h). After antigen coating, the wells were blocked with 250 μl of casein 0.2% in PBS for 4 h at 37°C and then washed four times with PBS containing 0.2% Tween 20. Immune or nonimmune sera were diluted in PBS containing 0.02% casein and 100 μl of each dilution was added to duplicate wells. After each well was washed four times, bound antibody was detected with horse radish peroxidase (Sigma Chemical Comp., St. Louis, MO) labelled goat anti-species specific IgG (37°C, 1 h), using O-phenylenediamine as the chromogen. Mean of duplicates OD (absorbance at 490 nm) of each treatment group was obtained and these data were compared on the basis of the inverse of the highest serum dilution that produced an OD reading four times above the negative control wells. For negative controls, antigen or serum was omitted from the wells.

Superantigen binding and TCR subset analysis.

Cells from the mouse B-lymphoma line A20 (ATCC, Rockville, MD)(2-4 x 10^5 cells) were incubated (40 min

at 37°C) with WT or mutant toxin in Hanks balanced salt solution containing 0.5% bovine serum albumin (HBSS, USAMRIID). The cells were washed with HBSS and incubated with 5 μg of affinity-purified anti-toxin antibody in HBSS (4°C, 45 min). Unbound antibody was removed and the bound antibody was detected with fluorescein isothiocyanate (FITC)-labelled, goat anti-rabbit IgG (Organon Teknika Corp., Durham, NC). Unbound antibody was removed and the cells were analyzed by with a FACSort flow cytometer (Becton Dikinson & Co. , Mountain View, CA).

For TCR subset analysis, splenic mononuclear cells were obtained from mice immunized with WT or mutant toxin. The mononuclear cells were incubated (37°C) with WT toxin (100 ng/mL) for 5 days and then cultured in 85% RPMI-1640, 10% interleukin-2 supplement (Advanced Biotechnologies Inc., Columbia, MD) with 5% FBS for an additional 5 days. The T cells were washed twice and stained with anti-TCR (Biosource, Camarillo, CA) or anti-Vß specific TCR (Biosource, Camarillo, CA) (45 min, 4°C). All cells analyzed were positive for T cell marker CD3+ and expressed the CD25 activation marker (data not shown). Controls were incubated with an isotype matched antibody of irrelevant specificity. Unreacted antibody was removed, and the cells were incubated with an FITC-labelled, anti-mouse IgG (Organon Teknika Corp, Durham, NC) on ice for 30 min. The cells were washed and analyzed by flow cytometry (FACSort).

<u>LPS potentiation of SE toxicity in mice.</u>

C57BL/6 or BALB/c mice weighing 18-20 g (Harlan Sprague Dawley, Inc., Frederick Cancer Research and Development Center, Frederick, MD) were each injected intraperitoneally (i.p.) with 200 μl of PBS containing

varying amounts of SEA, SEB, or SEC1, TSST-1, or SPEa followed 4 h later with 75 or 150 μg of LPS (200 μl/i.p.). Controls were each injected with either SE (30 mg) or LPS (150 mg). Animals were observed for 72 h after the LPS injection. Calculations of LD50 were done by Probit analysis using 95% fiducial limits (SAS Institute Inc., Cary, NC).

The biological effects of SEA and SEB were also tested in transgenic C57BL/6 mice (GenPharm International, Mountain View, CA) deficient in MHC class I or II expression [Stiles *et al.* (1993) *Infect. Immun.* **61**, 5333-5338], as described above, using a single dose of toxin (30 μg/mouse). Genetic homozygozity was confirmed by Southern analysis of parental tail DNA, using β2 microglobulin and MHC class II β DNA probes.

Detection of cytokines in serum.

Mice (n=18 per group) were injected with toxin (10 μg), LPS (150 μg), or toxin plus LPS. Sera were collected and pooled from three mice per group at each time point (2, 4, 6, 8, 10, 22 h) after LPS injection. Sera were collected at various time points following toxin injection (-4 h, or 4h before LPS injection, for data tabulation). Collection of LPS control sera began at the time of injection (0 h).

Serum levels of TNFα and IL-α were detected by an enzyme linked immunosorbent assay (ELISA). TNFα was first captured by a monoclonal antibody against mouse TNFα (GIBCO-BRL, Grand Island, NY) and then incubated with rabbit anti-mouse TNFα antibody (Genzyme, Boston, MA). The ELISA plate was washed and peroxidase conjugate of anti-rabbit antibody (Boehringer Mannheim, Indianapolis, IN) added to the wells. After washing the plate and adding substrate (Kirkegaard and

Perry, Gaithersburg, MD), TNFα concentrations were measured using the mean A450 reading of duplicate samples and a standard curve generated from recombinant mouse TNFα (GIBCO-BRL). Serum levels of IL-1α were determined from the mean reading of duplicate samples with an ELISA kit that specifically detects murine IL-1α (Genzyme, Boston, MA). The standard error of the mean (SEM) for TNFα and IL-1α readings was +/- 5%.

Quantitation of IL-6 and IFNγ were measured by bioassays [See *et al.*(1990) *Infect. Immun.* **58**: 2392-2396]. An IL-6 dependent cell line, 7TD1 (kindly provided by T. Krakauer), was used in a proliferative assay with serial two-fold dilutions of serum samples assayed in triplicate. Proliferation of 7TD1 cells in a microtiter plate was measured by uptake of [^{3}H]-thymidine (1 μCi/well; Amersham, Arlington Heights, IL) and the activity of IL-6 from serum was compared to a recombinant mouse IL-6 standard (R and D Systems, Minneapolis, MN) as previously described [See *et al.*(1990) *Infect. Immun.* **58**: 2392-2396]. The SEM of triplicate samples was +/- 10%.

IFNγ was measured by the reduction of vesicular stomatitis virus (New Jersey strain) cytopathic effects on L929 cells, as previously described [Torre *et al.*(1993) *J. Infect. Dis.* **167**, 762-765]. Briefly, serial two-fold dilutions of serum were made in duplicate and added to microtiter wells containing L929 cells (5 x 10^4/well). After incubating 24 h, virus (5 x 10^5 PFU/well) was added and the cytopathic effects measured at 48 h by absorbance readings (570 nm) of reduced 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (Sigma). The activity of each serum sample was determined using recombinant

mouse IFNγ as a standard (Biosource, Camarillo, CA). The SEM of duplicate samples was +/- 5%.

EXAMPLE 1

Molecular modelling and structural studies of staphylococcal and streptococcal superantigens: bacterial superantigens share common 3-dimensional structure.

Comparison of amino acid sequences (**Fig. 1**) suggested that bacterial superantigens fall into groups consisting of (1) SEA, SED and SEE, (2) SEB, staphylococcal enterotoxins C1-C3 (SEC1-3), the streptococcal pyrogenic exotoxins A (SPE-A) and C (SPE-C), (3) TSST-1 and (4) the exfoliative toxins (ETA, ETB) and streptococcal pyrogenic exotoxin B (SPE-B), which are the most distant from the others in sequence. Although not available to the inventor when the inventions were first conceived and proof of principle was obtained, the x-ray crystallographic structures of several bacterial superantigens are now known. Diverse superantigens, such as SEB and TSST-1, appear to have little sequence in common, yet they exhibit homologous protein folds composed largely of β strands [Prasad, G.S. *et al.* (1993) *Biochemistry* **32**, 13761-13766; Acharya, R.K. *et al.* (1994) *Nature* **367**, 94-97; Swaminathan, S. *et al.* (1992) *Nature* **359**, 801-806]within two distinct domains. Differences between the proteins are located primarily in highly variable regions comprised of several surface loops, such as the disulfide-bonded loop which is absent from TSST-1 and at the amino terminus.

The X-ray crystal structures of SEB and TSST-1 complexed with HLA DR1 are known [Kim, J. *et al.* (1994) Science 266, 1870-1874 ; Jardetzky, T.S. *et al.*

(1994) Nature 368, 711-718] and this data was useful to fully explain our results concerning attenuation of the superantigens by site-specific mutagenesis. The region of HLA DR1 that contacts SEB consists exclusively of α subunit surfaces. The main regions of SEB involved are two conserved sites: a polar pocket derived from three β strands of the β barrel domain and a highly solvent-exposed hydrophobic reverse turn. The polar binding pocket of SEB contains a glutamate and two tyrosines that accommodate Lys39 of the α subunit of HLA DR1, while the hydrophobic region consists of a leucine and flanking residues that make several contacts with the HLA DRα chain. The HLA DR1 binding sites of both TSST-1 and SEB overlap significantly. The hydrophobic binding contacts of other SAg with the HLA DRα chain have been proposed [Ulrich *et al.* (1995) *Nature*, Struct. Biol. **2**, 554-560] to be similar to those found in SEB and TSST-1. A motif consisting of a leucine in a reverse turn [Ulrich *et al.* (1995), supra] is conserved among bacterial superantigens and may provide the key determinant (hydrophobic or otherwise) for binding HLA-DR. However, TSST-1 does not have a highly charged residue in the polar pocket that interacts with Lys39 of the HLA DRα chain and uses an alternative conformational binding mode that allows TSST-1 to interact with HLA DR1 β -chain residues and the carboxy-terminal region of the antigenic peptide.

Both SEA and SEE bind to the β subunit of DR by means of a single zinc atom [Fraser, J.D. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5507-5511]. The amino-terminal domain of SEA interfaces with the HLA DRα chain [Ulrich *et al.* (1995), supra], while SEA C-terminal domain residues His187, His225 and Asp227 form a zinc-coordination complex, likely with His-81

from the β chain of an adjoining HLA DR molecule. However, our results have shown that binding of superantigen to the HLA DRβ subunit does not directly stimulate T cells [Ulrich *et al.* (1995), supra] but increases the potential of the bound SEA to interact with the α chain of another HLA DR, thus increasing the biological potency.

EXAMPLE 2

<u>Molecular modelling and structural studies of staphylococcal and streptococcal superantigens: A detailed protein structure analysis of SEB and SEA suggested that all bacterial superantigens have a common mechanism for binding MHC class II receptors.</u>

A least-squares superimposition of the unbound molecules of modeled SEA and the crystal structure of SEB, aligned according to their structurally conserved α-helical and ß-strand regions, exhibited a global folding pattern which is very similar. Differences between the two structures are calculated to be located primarily in loops of low sequence homologies, with the largest positional deviations occurring between structurally conserved regions of residues 18-20, 30-32, 173-181, 191-194, and the cysteine-loop region (90-111). Only one of these regions in SEB makes significant contact (residue Y94 in particular) with the HLA-DR1 molecule [Jardetzky, T.S. *et al.* (1994) *Nature* **368**, 711-718].

The binding interface between SEB and HLA-DR1 consists principally of two structurally conserved surfaces located in the N-terminal domain: a polar binding pocket derived from three ß-strand elements of the ß-barrel domain and a hydrophobic reverse turn. The binding pocket of SEB contains residues E67, Y89

and Y115, and binds K39 of the DRα subunit. For SEA, the binding interface with the DR molecule is modeled to contain a similar binding pocket consisting of residues D70, Y92 and Y108. Mutation of residue Y89 in SEB or Y92 in SEA to alanine (**Fig. 2**) resulted in 100-fold reduction in DR1 binding. The substitution of alanine for Y89 in SEB and Y92 in SEA eliminates the hydrogen bond with K39 and disrupts packing interactions with adjacent protein residues. Modeling of the SEA mutant Y92A predicts an increase in solvent-accessible surface area for Y108 by a factor of two greater than the wild-type structure, allowing the formation of a hydrogen bond to the carboxylate group of D70 and thus disrupting key anchoring and recognition points for HLA-DR1. This effect is expected to be somewhat less in SEB due to the longer side chain at E67. Substitution of SEB Y115 with alanine also resulted in 100-fold reduction of binding. In contrast, the same replacement of Y108 in SEA yielded little to no change in DR1 binding (**Fig. 2a**), suggesting the primary importance of SEA residues Y92 and D70 for stabilizing interactions with K39. The K39 side chain of DRα forms a strong ion-pair interaction with the SEB E67 carboxylate group and hydrogen bonds with the hydroxyl groups of Y89 and Y115. Substitution of SEB E67 by glutamine reduced binding affinity by 100-fold (**Fig. 2**), reflecting the replacement of the strong ionic bond with a weaker hydrogen bond. To optimize ion-pair interactions of the analogous SEA site, the shorter carboxylate side chain of D70 is predicted to shift K39 of DRα, weakening interactions with SEA Y108. The substitution of alanine for SEA Y108 is thus more easily accommodated than the homologous substitution of SEB Y115, without loss in DR1 binding.

Comparisons of the polar pocket with other bacterial superantigens were then made. SEC1-3 and SPE-A have conserved the critical DR1 binding-interface residues (**Fig. 1**), and share with SEB and SEA secondary structural elements of the DR1-binding surfaces. Asparagine in SED (N70) replaces the acidic side chain present in SEA, SEB, SPE-A and SEC1-3. Accordingly, for SED the salt bridge of the polar pocket is likely to be replaced by a hydrogen bond. Overall DR1 affinities for SED and SEA appeared to be equivalent (**Fig. 2b**), indicating that other interactions may compensate for the absence in SED of the ion-pair found in the other superantigens. For the case of TSST-1, mutating DRα residues K39 to serine or M36 to isoleucine has been shown to greatly reduce binding [Panina-Bordignon *et al.* (1992) *J. Exp. Med.* **176**: 1779-1784]. Although primarily hydrophobic, the critical TSST-1 structural elements are conserved with the SEA and SEB polar binding pocket. SEB residues Y89 and Y115 are homologous to T69 and I85 in TSST-1, respectively, and SEB E67 is replaced by I46. These TSST-1 residues are positioned in a conserved ß-barrel domain found in both SEB and SEA. However, the TSST-1 site lacks polarity equivalent to SEB/SEA, and hydrogen bonding with the hydroxyl of TSST-1 residue T69 would require that DRα K39 extend 5 Å into the pocket. TSST-1 binding utilizes an alternative strategy [Kim *et al.* (1994) *Science* **266**: 1870-1874] consisting of hydrophobic contacts centered around residue I46, and potential ionic or hydrogen bonds bridging DRα residues E71 and K67 to R34 and D27, respectively, of TSST-1.

The hydrophobic region of the binding interface between SEB and the HLA-DR1 molecule consists of SEB residues 44-47, located in a large reverse turn

connecting ß-strands 1 and 2 of SEB. These residues appear to make strong electrostatic interactions with DRα through their backbone atoms. The mutation of L45 to an arginine reduced overall HLA-DR1 binding greater than 100-fold (**Fig. 2b**), attributable to the less energetically favorable insertion of a highly charged residue into a hydrophobic depression on the DR1 molecule. The modeled DR1-SEA complex presents similar interactions with the SEA backbone atoms, with the exception of a glutamine (Q49) replacing SEB Y46. Mutation of L48 to glycine in SEA (homologous to L45 of SEB) has been reported to decrease T-cell responses. SEB L45 and the comparable L30 of TSST-1 are the most extensively buried residues in the DR1 interface. The leucine is conserved among the bacterial superantigens (**Fig. 3**) and may provide the necessary hydrophobic structural element for surface complementarity with DR1, consistent with the mutagenesis data for SEB and SEA.

The inventor has performed similar structure and function studies with TSST-1, SEC1 and SPE-A.

EXAMPLE 3

<u>Molecular modelling and structural studies of staphylococcal and streptococcal superantigens: Some interactions of bacterial superantigens with MHC class II receptors are not conserved but are less important than the hydrophobic loop and polar pocket binding sites.</u>

In determining the overall affinity of the superantigen for DR1, a contributory role is played by structural variations around the common binding motifs. A short, variable structured, disulfide-bonded loop is found in SEA and a homologous longer

loop in SEB. The SEB residue Y94, contained within this loop, forms hydrophobic interactions with L60 and A61 of the DRα subunit. Replacement of Y94 with alanine partially inhibits DR1 binding (**Fig. 2a,b**). An alanine is found in SEA (A97) and SEE at the position equivalent to SEB Y94, and mutating this residue in SEA to tyrosine results in disrupted instead of stabilized interactions with DR1 (**Fig. 2a**). Although the disulfide loops differ in structure between SEA and SEB, A97 apparently contributes to the DRα binding interface in a manner similar to Y94 of SEB. Because TSST-1 lacks a disulfide loop, similar contacts with DRa are replaced by interactions with β-strands of TSST-1. In a like manner, the absence of a salt bridge between the residues K39 of DRα and E67 of SED is apparently compensated for by stabilizing interactions occurring outside of the otherwise conserved dominant binding surfaces (**Fig. 2a**).

EXAMPLE 4

<u>Molecular modelling and structural studies of staphylococcal and streptococcal superantigens: Superantigen interactions with T-cell antigen receptors.</u>

The amino acid residues in contact with TCR are located in regions of high sequence variability, presenting a unique surface for interaction with the TCR. Residues implicated in TCR interactions by mutagenesis of SEA and SEB reside in variable loop regions, while TSST-1 mutants that affect TCR binding are mainly located in an α helix [Acharya, R.K. *et al.* (1994) *Nature* **367**, 94-97; Kim, J. *et al.* (1994) *Science* **266**, 1870-1874]. Specifically, mutations that diminish T-cell receptor recognition of SEB include

residues N23, Y61, and the homologous SEA N25 or Y64 **(Fig. 2c)**. SEA residues S206 and N207 also control T-cell responses [Hudson, *et al.*(1992) *J. Exp. Med.* **177**: 175-184]. Mutants of the polar binding pocket, SEA Y92A and SEB Y89A, equivalently reduced T-cell responses (**Fig. 2c**), reflecting the observed decreases in DR1-binding (**Fig. 2a, b**). While supporting reduced T-cell responses, mutants SEA Y64A and SEB Y61A retained normal affinities for DR1 (**Fig. 2a-c**).

EXAMPLE 5

Animal models for determining biological activity of bacterial superantigens: Mouse.

When compared to primates, mice are not very susceptible to the toxic effects of SE, and we therefore sought to increase sensitivity with a potentiating dose of lipopolysaccharide (LPS) from Gram-negative bacteria [Stiles *et al.* (1993) *Infect. Immun.* **61**, 5333-5338]. There was no apparent effect in control animals injected with any of the SE (up to 30 μg/mouse) or LPS (150 μg/mouse) alone (Table 1). Incremental injections of LPS were also not lethal, when given in amounts up to 250 μg/mouse (data not shown). However, mice died between 24-48 h after SE and LPS were given to the same animal (Table 1). SEA was much more toxic than either SEB or SEC1 and the calculated LD50 (μg toxin/kg) of SEA, SEB, and SEC1 with 95% fiducial limits was 18.5 (6.5, 38.5), 789.0 (582.5, 1044.5), and 369.0 (197.5, 676.0), respectively.

TABLE 1. Titration of SEA, SEB, and SEC_1 in the C57BL/6 mouse lethality assay

Stimulus[a]	% Lethality (no. of mice tested) with the following dose of SE, in micrograms/mouse[b]:			
	30	10	1	0.1
SEA + LPS	93(15)[b]	85(20)	80(15)	20(10)
SEB + LPS	80(15)	27(15)	0(15)	0(15)
SEC_1+ LPS	80(10)	60(10)	10(10)	0(10)

[a]LPS was injected into each mouse (150ug) 4 h after the SE injection. Control mice injected with 150 ug of LPS (n=20) or 30 ug of SEA, SEB, or SEC1 (n=10) survived.

[b]Results are from a combination of separate experiments with five mice per experiment.

The role of MHC class I and class II molecules in SE toxicity, potentiated by LPS, was addressed by using transgenic, MHC-deficient mice (Table 2). Class II-deficient animals were unaffected by a dose of SE (30 µg) plus LPS (150 µg) that was lethal for 93% of wild-type and 30% of class I-deficient mice. Mononuclear cells from class II-deficient animals were not able to present SEA, as measured by proliferative responses. MHC class I-deficient cells were functional in supporting T-cell proliferation, but at levels <30% of the proliferative response supported by MHC-wild-type presenting cells (Table 3). Cell surface expression levels were normal, when compared to nontransgenic C57BL/6, for A^b in class I-deficient mice, and K^b/D^b in class II-deficient mice. The T-cell responses of MHC class I- or class II-deficient mice were essentially equivalent to wild-type when SEA

was presented by mononuclear cells expressing both class I and II molecules (Table 3).

TABLE 2. Lethality of SEA and SEB in C57BL/6 mice lacking MHC class I or class II

Stimulus[a]	% Lethality (no. of mice tested) with the following MHC class phenotype		
	I^-II^+	I^+II^-	I^+II^+
SEA + LPS	30(10)	0(5)	93(15)
SEA + LPS	ND[b]	0(5)	80(15)
SEA only	0(2)	0(2)	0(2)
SEB only	ND[b]	0(2)	0(2)
LPS only	0(5)	0(5)	0(5)

[a] Mice were injected with 30 ug of SEA or SEB and, 4h later, with 150 ug of LPS, as indicated. Control mice were injected with only SEA, SEB, or LPS.

[b] ND, not determined.

Table 3. Mouse T-cell responses to SEA are MHC class II-dependent

T-cell/APC source[3]	T-cell responses[1] 0.1 µg/ml SEA	1 µg/ml SEA
Wild-type C57/BL6 mouse/autologous	430,000 cpm[2]	700,000 cpm
MHC class I knock-out C57/BL6 mouse/autologous	117,000 cpm	167,000 cpm
MHC class II knock-out C57/BL6 mouse/autologous	8,000 cpm	33,000 cpm
Wild-type C57/BL6 mouse/wild-type	305,000 cpm	307,000 cpm
MHC class I knock-out	420,000 cpm	445,000 cpm

C57/BL6 mouse/wild-type		
MHC class II knock-out C57/BL6 mouse/wild-type	310,000 cpm	322,000 cpm

[1]Cultures of mononuclear cells derived from mouse spleens, cultured for 3 d with the indicated amount of SEA.

[2]Data represent the mean of triplicate determinations (<10 SEM) of $[^3H]$thymidine incorporation.

[3]Antigen presenting cells (APC) were isolated from spleens of the indicated mouse strain and added to cultures.

The serum levels of TNFα, IL-1α, IL-6, and IFNγ in mice injected with SEA, LPS, or SEA plus LPS were measured at various times following injection (**Fig. 4**). Compared to mice injected with either SEA or LPS alone, the serum levels of TNFα, IL-6, and IFNγ had increased 5-, 10-, and 15-fold, respectively, in animals given SEA plus LPS. SEA alone did not elicit any detectable increase of TNFα, IL-6, or IFNγ above background. In contrast to the other cytokines, IL-1α levels in mice injected with SEA plus LPS resulted in a simple additive effect.

Serum levels of TNFα, IL-6, and IFNγ were maximal 2-4 h after the LPS injection, but returned to normal by 10 h. The concentration of IL-1α in mice given SEA plus LPS had also peaked 2 h after the LPS injection, but stayed above background for the remaining determinations. Levels of IL-1α in mice given only LPS or SEA peaked at 4 and 6 h, respectively. Unlike profiles for other cytokines, the highest amount of IL-1α in mice injected with SEA and LPS corresponded to the peak stimulated by SEA, but not LPS.

This animal model was used in various stages of developing the inventions, as a means of assessing the physiological activity of mutated superantigens.

Control animals survived the maximum dose of either SE or LPS, while mice receiving both agents died. Wild-type SEA was 43-fold more potent than SEB and 20-fold more potent than SEC1. By using BALB/c mice the toxicity of SEB was 10-20 fold higher. These data confirmed that the toxicity of SE was mainly exerted through a mechanism dependent on expression of MHC class II molecules and was linked to stimulated cytokine release. Thus this was a relevant preclinical model that could be used to predict human responses.

EXAMPLE 6

Animal models for determining biological activity of bacterial superantigens: Rhesus monkey

The physiological responses of the rhesus monkey to bacterial superantigens is probably identical to humans, with the exception of sensitivity [Bavari and Ulrich (1995) *Clin. Immunol.Immunopath.* **76**:248]. Generally SEB intoxicated monkeys developed gastrointestinal signs within 24 hours post-exposure. Clinical signs were mastication, anorexia, emesis and diarrhea. Following mild, brief, self-limiting gastrointestinal signs, monkeys had a variable period of up to 40 hours of clinical improvement. At approximately 48 hours post-exposure, intoxicated monkeys generally had an abrupt onset of rapidly progressive lethargy, dyspnea, and facial pallor. If given a lethal dose, death occurs within four hours of onset of symptoms. Only SEB has been used in challenges of rhesus monkeys to determine physiological/pathological effects. Human responses to bacterial superantigens are characterized by a rapid drop in blood pressure, elevated temperature,

and multiple organ failure-the classical toxic shock syndrome (TSS). However, the respiratory route of exposure may involve some unique mechanisms. The profound hypotension characteristic of TSS is not observed, and respiratory involvement is rapid, unlike TSS. Fever, prominent after aerosol exposure, is generally not observed in cases of SEB ingestion.

EXAMPLE 7

Targeting receptor interactions to develop vaccines.

The SEA mutants Y92A, with reduced DR1 binding, and Y64A, with reduced TCR interactions, and K14E with wild-type (control) activity were used to determine the correct receptor to target for vaccine development. The binding of WT or mutant SEA was evaluated with the MHC class II expressing murine B-cell lymphoma cell line A20 (Table 4). The binding affinity of WT SEA to mouse MHC class II ($H-2^d$) molecules was lower than that observed with human MHC class II expressing cells , reflecting the reduced toxicity that bacterial SAgs exert in mice. WT SEA, Y64A and K14E all had the same relative affinity to mouse MHC class II molecules. Similar to the results obtained with human MHC class II molecules, the Y92A mutant exhibited substantially reduced binding to A20 cells (Table 4).

Table 4. Biological activity of superantigen vaccines

toxin	T-cell anergy[1]	MHC classII binding[2]	T-cell response
SEA wild type	++++	+++	+++
TCR attenuated Y64A	+	+++	+/-
MHC attenuated Y92A	-	+/-	+/-
Control K14E	++++	+++	+++

[1]Based on attenuation of T-cell response to wild-type SEA in mice immunized with the mutant or wild-type SEA.

[2]Binding to the mouse MHC class II+ A20 cells, measured by flow cytometry

The effect of WT SEA or site-specific SEA mutants on splenic mononuclear cells obtained from nonimmunized C57BL/6 (H-2^b) mice is summarized in Table 4. Both WT SEA and the control mutant K14E were potent T cell activators, effective at minimal concentrations of 10 to 100 pg/mL. However, T-cell responses to Y92A were reduced at least 100-fold, compared to SEA wild type, while Y64A-stimulated responses were slightly higher than Y92A. These results confirmed that attenuation of superantigen binding to either MHC class II or TCR molecules resulted in dramatically reduced mouse T-cell proliferation. These results may indicate that the altered toxin may compete with wild type toxin for TCR binding.

SEA WT (10 LD50), site-specific SEA mutants (10 μg/mouse each) or LPS (150 μg/mice) injected alone were nonlethal to mice (Table 5). However, combining

LPS with either WT SEA or mutant K14E resulted in 100% lethality. For those mice receiving both LPS and WT or K14E SEA, 80% were dead by 24 h and 100% by 48 h. In contrast, 100% of Y92A and 80% of Y64A injected mice (coadministered with LPS) survived. The average time to death for the 20% of mice that did not survive Y64A injection occurred at 48 to 72 h. These *in vivo* data correlated well with the results obtained with the lymphocyte cultures. It was concluded that the observed attenuation of toxicity in mice was a direct result of the reduced T-cell proliferation.

<u>Table 5. Biologic effect of wild type (WT) staphylococcal enterotoxin A (SEA) and SEA mutants</u>.

Protein	No. live/total
WT	0/10
K14E	0/10
Y64A	8/10
Y92A	10/10

NOTE. Mice were given 10 LD_{50} (10ug) of WT or mutant SEA. Lipopolysaccharide (150 ug/mouse) was injected 3 h later.

Having established that attenuation of receptor binding resulted in reduced toxicity, we next examined the immunogenicity of the SEA mutants. Mice were immunized with WT or mutant SEA. Control mice received adjuvant only or were left untreated. One week before challenge with WT SEA, mice were bled and serum antibody titers were determined for each group (Table 6). Mice immunized with the 2 μg of Y64A or Y92A had serum antibody titers of 1:5000 and 1:1000, respectively. Immunization with 2 μg of WT SEA or

control mutant resulted in titers of 1:5,000 and 1:10,000, respectively. The highest immunizing dose (10 μg/mouse) was most effective for all animals, resulting in antibody titers which were greater than 1:10,000. All mice were challenged with 10 LD50 of WT SEA (potentiated with LPS). The survival data correlated well with the levels of serum antibodies in immunized mice. All mice that were vaccinated with 10 μg of Y64A or Y92A, survived the lethal challenge dose of WT SEA. Slightly less protection was afforded by the lower vaccination dose of mutant Y64A or Y92A. All mice immunized with both doses of WT SEA survived the lethal challenge with WT potentiated with LPS. Mice immunized with mutant K14E exhibited survivals of 100% and 80% for high and low vaccination doses, respectively. All nonimmunized or control mice that were vaccinated with adjuvant alone died when challenged with WT SEA and a potentiating dose of LPS.

Table 6. Mice immunized with attenuated forms of staphylococcal enterotoxin A (SEA) produce high titers of neutralizing antibody.

Immunizing agent	Dose (ug/mouse)	Anti-SEA antibody titer*	No. live/total
WT	2	10,000-50,000	10/10
	10	10,000-50,000	10/10
K14E	2	5,000-10,000	8/10
	10	10,000-50,000	10/10
Y64A	2	5,000-10,000	6/10
	10	10,000-50,000	10/10
Y92A	2	1,000-5,000	2/10
	10	10,000-50,000	10/10
Adjuvant		50-100	0/10

NOTE. Mice were given 10 LD_{50} of wild type (WT) SEA challenge followed by potentiating dose of lipopolysaccharide (150 ug/mouse) 3 h later.

*Reciprocal of serum dilution resulting in optical density reading four times above negative controls (wells containing either no SEA or no primary antibody).

EXAMPLE 8

Immune recognition of SAg mutants.

Bacterial SAgs induce clonal anergy of specific subsets of T cells in mice. It was possible that the loss of sensitivity to WT SEA among the mice vaccinated with the attenuated mutant forms represented a state of specific non-responsiveness instead of specific immunity. To address this issue, lymphocyte responses to SEA WT were measured with splenic mononuclear cells collected 2 weeks after the third immunization. As expected, lymphocytes from mice that were immunized with WT SEA or control SEA mutant showed little to no proliferation when incubated with the WT SAg. In contrast, lymphocytes obtained from control mice or those immunized with either Y64A or Y92A all responded vigorously to the WT SEA (**Fig. 5**). The TCRs used by T cells from the SEA-vaccinated mice were then characterized by flow cytometry. T cells from immunized or control mice were incubated with WT SEA in culture for 7 days, followed by a 5 day expansion in IL-2 containing medium. Distinct populations of activated TCR Vß11 positive cells were observed with T cells from mice immunized with Y92A and Y64A, representing 48% and 40% of T cells, respectively. However, Vß11 expressing cells obtained from SEA WT or K14E immunized mice were about 1% and 6% of the total T-cell population, respectively, suggesting that this subset was

nonresponsive to restimulation with the WT SAg. T cells bearing Vß 17a, 3, 7, and 10b were unchanged for all mice. It was apparent that T-cell responses to both the TCR and MHC class II binding-attenuated SEA mutants were similar to each other, but differed from responses to control or WT molecules. These results suggested that an alternative, perhaps conventional antigen processing mechanism was functioning in presentation of the SAg mutants Y64A and Y92A.

EXAMPLE 9

Rhesus monkey immunizations with monovalent vaccines.

The SEA vaccine L48R, Y89A, D70R (A489270) and SEB vaccine Y89A, Y94A, L45R (B899445) were used to immunize rhesus monkeys. The animals received a total of three i.m. injections (10-20 μg/animal), given at monthly intervals. Rhesus monkeys that were injected with these vaccines had no detectable increase of serum cytokines and no apparent toxicity. The serological response of animals vaccinated with three doses of formalin-treated SEB toxoid (100 μg/injection) gave results comparable to one or two injections with B899445 (Table 7), suggesting that the recombinant vaccines were very immunogenic. Immunized rhesus monkeys survived a lethal challenge with >10 LD50 of wild-type SEB (Table 7, 8). Collectively, these results suggest that the engineered SEB vaccine is safe, highly antigenic and effective at protecting the immunized individual from lethal aerosol exposure to SEB.

Table 7. Rhesus monkey antibody responses to vaccine B899445; One injection of B899445 outperforms three injections of SEB toxoid

Vaccine[1]/animal #	Antibody response[2]	%Inhibition of T-cell response[3]	Survival SEB >20 x LD50 challenge[4]
preimmune sera /pooled	0.161	5	dead
toxoid/1	0.839	0	dead
toxoid/2	0.893	34	live
toxoid/3	1.308	57	live
toxoid/4	1.447	55	live
B899445/1	1.788	69	live
B899445/2	0.78	49	live

[1]Rhesus monkeys were immunized with one dose (20 μg injection) of B899445 vaccine or three doses of formalin-treated SEB toxoid (100 μg/injection) one month apart; both used Alum adjuvants.

[2]Sera were collected one month after the final injection. Antibody responses were determined by ELISA and the results are shown as mean optical densities of triplicate wells (± SEM).

[3]Rhesus monkey T cells, obtained from an untreated animal, were preincubated with diluted (1:70) serum from immunized monkeys and then cultured with wild type SEB. Data are shown as % of T cell responses, where serum of rhesus monkey injected with adjuvant only represented the 100% of response to wild type SEB.

[4]Rhesus monkeys were challenged by aerosol exposure and monitered for four days.

Table 8. Engineered staphylococcal enterotoxin B vaccine efficacy in rhesus monkeys

Treatment[1]	Antibody titer[2]	Immune protection[3]
Vaccine with adjuvant	>10,000	100%
Adjuvant only	<50	0%

[1]Rhesus monkeys (n=10) were injected i.m. with 10 µg of SEB vaccine with Alhydrogel adjuvant. A total of 3 immunizations, 1 month apart were given. Controls (n=2) received only Alhydrogel.

[2]Serum dilution resulting in optical density readings of four times above the negative control, consisting of no SEB or serum added to the wells.

[3]Immunized and control rhesus monkeys were challenged with >10 LD50 of wild-type staphylococcal enterotoxin B as an aerosol.

Serum from monkeys that were immunized with the genetically attenuated vaccine inhibited T-lymphocyte responses to wild type SEB (Table 7) similarly or better than monkeys that received the SEB toxoid. Collectively, these results suggest that the recombinant SAg vaccines are safe, highly antigenic, and induce protective immunity.

Serum from B899445 immunized rhesus monkeys blocked human lymphocyte responses to wild-type superantigen when tested in *ex vivo* cultures (Table 7). These data again showed that the second and third injections of vaccine were approximately equivalent in stimulating neutralizing antibody responses. Normal T-cell responses to several superantigens, including the wild-type protein, were observed in immunized animals, indicating that no specific or generalized anergy occurred (**Fig. 6**).

EXAMPLE 10

A. Multivalent superantigen vaccines: Rhesus monkey immunizations.

Rhesus monkeys were immunized with a combined vaccine consisting of B899445 and A489270. Following the third injection, antibody recognition of wild-type bacterial superantigens was examined (**Fig.7**). High titers of anti-SEB, SEC1 and SEA antibodies were evident.

B. Mouse immunizations.

Mice (BALB/c) were immunized with a combined vaccine consisting of SEA, SEB, SEC1 and TSST-1 (all wild-type). The antibody responses against each individual superantigen were assessed (Table 9). Antibodies were induced against each of the component antigens, providing sufficient levels to protect the mice from a lethal challenge of superantigen, potentiated with LPS. Although not shown in the Table, antibody responses against SPE-A were also observed. Mice were also immunized with individual superantigens and antibody responses against other superantigens were measured (Table 10). Each individual immunogen induced partial or complete protective antibody responses against all other superantigens tested.

TABLE 9. Superantigen cross-reactivity of antibodies from mice immunized with individual bacterial superantigens

Immunizing[1] Toxin	Challenging[2] Toxin	ELISA[3] Titer	Neutralizing[4] Antibody
SEA	SEA	>1/25,000	100%
SEA	SEB	>1/25,000	100%
SEA	SEC1	>1/25,000	100%
SEA	TSST1	>1/10,000	100%
SEB	SEB	>1/25,000	100%
SEB	SEA	>1/10,000	100%
SEB	SEC1	>1/2,500	100%
SEB	TSST1	>1/10,000	100%
SEC1	SEC1	>1/10,000	100%
SEC1	SEA	>1/10,000	100%
SEC1	SEB	>1/25,000	100%
SEC1	TSST1	>1/10,000	100%
TSST1	TSST1	<1/10,000	100%
TSST1	SEA	<1/1,000	50%
TSST1	SEB	<1/1,000	40%
TSST1	SEC1	<1/1,000	40%

[1]Three injections with 20 ug of antigen (BALB/c mice).

[2]LPS-potentiated challenge with 10 LD_{50}s of superantigen.

[3]ELISA antibody response against an individual superantigen.

[4]Percent mice surviving an LPS-potentiated challenge (n=10).

Table 10. Multivalent superantigen vaccine. Mouse immune responses.

Immunizing toxin[1]	Challenging toxin[2]	Antibody Titer[3]	% survival
SE-A, B, C1, TSST-1	all	N/A	100%
" "	SEA	>25,000	100%
" "	SEB	>25,000	100%
" "	SEC1	>25,000	100%
" "	TSST-1	>6,400	100%

[1]Total of three injections, two weeks apart, in RIBI adjuvant.

[2]>10 X LD50, potentiated with *E. coli* lipopolysaccharide.

[3]Measured by ELISA.

EXAMPLE 11

Design of altered TSST-1 toxin vaccine, TST30.

A comprehensive study of the relationships of TSST-1 protein structure to receptor binding were undertaken to provide insight into the design of the vaccine TST30. We have discovered that TSST-1 interactions with the human MHC class II receptor, HLA-DR, are relatively weak and can be disrupted by altering only a single critical amino acid residue of the toxin. Site-directed mutagenesis of a gene encoding the toxin and expression of the new protein product in *E. coli* were then used to test the design of the vaccine. The TSST-1 gene used was contained within a fragment of DNA isolated by BglI restriction enzyme digestion of the gene isolated from a toxigenic strain of *Staphylococcus aureus* (AB259; Kreiswirth and Novick (1987) *Mol. Gen. Genet.* **208**, 84-87). The sequence of this gene is identical to all currently

known TSST-1 isolates of human origin. The wild-type TSST-1 gene can be readily cloned from a number of clinical S. aureus isolates. The DNA fragment containing the TSST-1 gene was isolated by agarose gel electrophoresis and ligated inot the prokaryotic expression vector pSE380 (Invitrogen Corp.). The DNA clone consisted of sequences encoding the leader peptide and the full length of the mature TSST-1 protein. This engineered vaccine is currently being evaluated to determine mouse and human T-cell reactivities in vitro, and lethality in mice. The TST30 vaccine consists of the following mutation introduced into the toxin molecule: leucine at amino acid residue 30 changed to arginine. Two other mutations, namely Asp27 to Ala and Ile46 to Ala have also been designed. The final vaccine may incorporate one or both of these additional mutations.

The binding interface between TSST-1 and HLA-DR consists of a large relatively flat surface located in the N-terminal domain. Leucine 30 protrudes from a reverse turn on the surface of TSST-1 and forms the major hydrophobic contact with the HLA-DR receptor molecule. Mutation of the single residue leucine 30 in TSST-1 to the charged amino acid side chain of arginine is predicted to disrupt this major contact with the receptor molecule, resulting in a significant reduction in DR1 binding. This mutant molecule should therefore have lost the toxin attributes of the wild-type molecule.

TST30 was expressed as a recombinant protein in E.coli, as either a periplasmically secreted protein or as a cytoplasmic product. Purification was achieved by immunoaffinity chromatography or preparative isoelectric focusing after an initial ion-exchange CM-Sepharose enrichment step. The method of

purification was not critical to the performance of the vaccine. Lipopolysaccharide contaminants, resulting from expression in a Gram-negative bacterium, were readily removed (as determined by limulus assay) using a variety of standard methods. The final purified vaccine is not toxic to mice at levels equivalent to 10 LD_{50} of the native TSST-1. No indicators of toxicity were found in surrogate assays of human T-cell stimulation.

Conclusive vaccine studies demonstrating that TST30 is highly antigenic and induces protective immunity are in progress in a mouse animal model. Mouse lethality is achieved at less than 1 ug/animal when a potentiating signal like lipopolysaccharide from Gram-negative bacteria (LPS) is provided. When coadministered with LPS, wild-type TSST-1 is 100% lethal to mice (10 LD_{50}). Mice receive three injections (two weeks between injections) of 20 ug/mouse in alhydrogel and protection against the lethal effects of 10 LD_{50} of TSST-1 are assessed.

EXAMPLE 12

Design of altered SPEA toxin vaccine, SPEa42

The SPEa interactions with human MHC class II receptor, HLA-DR, are relatively weak and can be disrupted by altering only a single critical amino acid residue of the toxin. Site-directed mutagenesis of a gene encoding the toxin and expression of the new protein product in E.coli were then used to test the design of the vaccine. The SPEa gene used was clone from a SPEa-toxigenic strain of *Streptococcus* by using specific DNA oligonucleotide primers and the polymerase chain reaction method. The sequence of this gene is identicla to SPEa isolates of human origin known within the public domain. The DNA

fragment containing the SPEa gene was isolated by agarose gel electrophoresis and ligated into a prokaryotic expression vector (pETx or pSE380). The DNA clone consisted of sequences encoding the leader peptide and the full length of the mature SPEa protein or SPEa42 without a leader sequence. We recognize that there are additional ways to express or produce the mature SPEa vaccine. The SPEa vaccine consists of the following mutation introduced into the toxin molecule: leucine at amino acid residue 42 changed to arginine.

The binding interface between SPEa and HLA-DR is predicted to consist of contacts located in the N-terminal domain that are conserved with other bacterial superantigens. Leucine 42 of SPEa is predicted to protrude from a reverse turn on the surface of SPEa and form a major hydrophobic contact with the HLA-DR receptor molecule. Mutation of the single residue leucine 42 in SPEa to the charged amino acid side chain of arginine is predicted to disrupt this major contact with the receptor molecule, resulting in a significant reduction in DR1 binding. This mutant molecule should therefore have lost the toxin attributes of the wild-type molecule.

SPEa42 was expressed as a recombinant protein in E.coli, as either a periplasmically secreted protein or as a cytoplasmic product. Purification was achieved by immunoaffinity chromatography or preparative isoelectric focusing after an initial ion-exchange CM-Sepharose enrichment step. The method of purification was not critical to the performance of the vaccine. Lipopolysaccharide contaminants, resulting from expression in a Gram-negative bacterium, were readily removed (as determined by limulus assay) using a variety of standard methods.

The final purified vaccine is not toxic to mice at levels equivalent to 10 LD_{50} of the native TSST-1. No indicators of toxicity were found in surrogate assays of human T-cell stimulation.

Conclusive vaccine studies demonstrating that SPEa42 is highly antigenic and induces protective immunity are in progress in a mouse animal model. Mouse lethality is achieved at less than 1 ug/animal when a potentiating signal like lipopolysaccharide from Gram-negative bacteria (LPS) is provided. When coadministered with LPS, wild-type SPEa is 100% lethal to mice (10 LD_{50}). Mice receive three injections (two weeks between injections) of 20 ug/mouse in alhydrogel and protection against the lethal effects of 10 LD_{50} of SPEa are assessed

EXAMPLE 13

Design of altered superantigen toxin vaccine, SEC45

For Staphylococcal enterotoxin C1 (SEC1), the leucine at position 45 was changed to lysine (SEC45). This mutation is anticipated to prevent SEC1 from interacting with the MHC class II receptor by sterically blocking the hydrophobic loop (centered around leucine 45) from binding to the alpha chain of the receptor. SEC1 is more closely homologous to SEB than SEA or the other superantigen toxins. The presence of zinc in SEC1 may impart additional binding characteristics that allow, in some cases, this superantigen toxin to bind to T-cell antigen receptors without the required MHC class II molecule interactions. To circumvent the binding to T-cell antigen receptors, mutations of SEC1 residues N23

(changed to alanine), V91 (changed to lysine) are being performed.

SEQUENCE LISTING

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Macintosh 7.5
(D) SOFTWARE: Microsoft Word 6.0

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(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

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SEQ ID NO:1:
Staphylococcal enterotoxin A vaccine A489270P, periplasmic
Gene sequence:

1 ATGAAAAAAA CAGCATTTAC ATTACTTTTA TTCATTGCCC TAACGTTGAC AACAAGTCCA

61 CTTGTAAATG GTAGCGAGAA AAGCGAAGAA ATAAATGAAA AAGATTTGCG AAAAAAGTCT

121 GAATTGCAGG GAACAGCTTT AGGCAATCTT AAACAAATCT ATTATTACAA TGAAAAAGCT

181 AAAACTGAAA ATAAAGAGAG TCACGATCAA TTTCGACAGC ATACTATATT GTTTAAAGGC

241 TTTTTTACAG ATCATTCGTG GTATAACGAT TTATTAGTAC GTTTTGATTC AAAGGATATT

301 GTTGATAAAT ATAAAGGGAA AAAAGTAGAC TTGTATGGTG CTTATGCTGG TTATCAATGT

361 GCGGGTGGTA CACCAAACAA AACAGCTTGT ATGTATGGTG GTGTAACGTT ACATGATAAT

421 AATCGATTGA CCGAAGAGAA AAAAGTGCCG ATCAATTTAT GGCTAGACGG TAAACAAAAT

481 ACAGTACCTT TGGAAACGGT TAAAACGAAT AAGAAAAATG TAACTGTTCA GGAGTTGGAT

541 CTTCAAGCAA GACGTTATTT ACAGGAAAAA TATAATTTAT ATAACTCTGA TGTTTTTGAT

601 GGGAAGGTTC AGAGGGGATT AATCGTGTTT CATACTTCTA CAGAACCTTC GGTTAATTAC

661 GATTTATTTG GTGCTCAAGG ACAGTATTCA AATACACTAT TAAGAATATA TAGAGATAAT

721 AAAACGATTA ACTCTGAAAA CATGCATATT GATATATATT TATATACAAG TTAAACATGG

781 TAGTTTTGAC CAACGTAATG TTCAGATTAT TATGAACCGA GAATAATCTA

SEQ ID NO: 2
Staphylococcal enterotoxin A vaccine A489270P, periplasmic:
Amino acid sequence

1 MKKTAFTLLL FIALTLTTSP LVNGSEKSEE INEKDLRKKS ELQGTALGNL

51 KQIYYYNEKA KTENKESHDQ FRQHTILFKG FFTDHSWYND LLVRFDSKDI

101 VDKYKGKKVD LYGAYAGYQC AGGTPNKTAC MYGGVTLHDN NRLTEEKKVP

151 INLWLDGKQN TVPLETVKTN KKNVTVQELD LQARRYLQEK YNLYNSDVFD

201 GKVQRGLIVF HTSTEPSVNY DLFGAQGQYS NTLLRIYRDN KTINSENMHI

251 DIYLYTS

SEQ ID NO: 3
Staphylococcal enterotoxin A vaccine A489270C, cytoplasmic
Gene sequence:

74 ATGAGAA AAGCGAAGAA ATAAATGAAA AAGATTTGCG
AAAAAAGTCT

121 GAATTGCAGG GAACAGCTTT AGGCAATCTT AAACAAATCT ATTATTACAA
TGAAAAAGCT

181 AAAACTGAAA ATAAAGAGAG TCACGATCAA TTTCGACAGC ATACTATATT
GTTTAAAGGC

241 TTTTTTACAG ATCATTCGTG GTATAACGAT TTATTAGTAC GTTTTGATTC
AAAGGATATT

301 GTTGATAAAT ATAAAGGGAA AAAAGTAGAC TTGTATGGTG CTTATGCTGG
TTATCAATGT

361 GCGGGTGGTA CACCAAACAA AACAGCTTGT ATGTATGGTG GTGTAACGTT
ACATGATAAT

421 AATCGATTGA CCGAAGAGAA AAAAGTGCCG ATCAATTTAT GGCTAGACGG
TAAACAAAAT

481 ACAGTACCTT TGGAAACGGT TAAAACGAAT AAGAAAAATG TAACTGTTCA
GGAGTTGGAT

541 CTTCAAGCAA GACGTTATTT ACAGGAAAAA TATAATTTAT ATAACTCTGA
TGTTTTTGAT

601 GGGAAGGTTC AGAGGGGATT AATCGTGTTT CATACTTCTA CAGAACCTTC
GGTTAATTAC

661 GATTTATTTG GTGCTCAAGG ACAGTATTCA AATACACTAT TAAGAATATA
TAGAGATAAT

721 AAAACGATTA ACTCTGAAAA CATGCATATT GATATATATT TATATACAAG
TTAAACATGG

781 TAGTTTTGAC CAACGTAATG TTCAGATTAT TATGAACCGA GAATAATCTA

SEQ ID NO:4
Staphylococcal enterotoxin A vaccine A489270C, cytoplasmic
Amino acid sequence

1 MEKSEE INEKDLRKKS ELQGTALGNL KQIYYYNEKA KTENKESHDQ

47 FRQHTILFKG FFTDHSWYND LLVRFDSKDI VDKYKGKKVD LYGAYAGYQC

97 AGGTPNKTAC MYGGVTLHDN NRLTEEKKVP INLWLDGKQN TVPLETVKTN

147 KKNVTVQELD LQARRYLQEK YNLYNSDVFD GKVQRGLIVF HTSTEPSVNY

197 DLFGAQGQYS NTLLRIYRDN KTINSENMHI DIYLYTS

SEQ ID NO: 5
Staphylococcal enterotoxin B vaccine, B2360210
Gene sequence:

1 GAACTAGGTA GAAAAATAAT TATGAGAAAA CACTATGTTG TTAAAGATGT

51 TTTCGTATAT AAGTTTAGGT GATGTATAGT TACTTAATTT TAAAAGCATA

101 ACTTAATTAA TATAAATAAC ATGAGATTAT TAAATATAAT TAAGTTTCTT

151 TTAATGTTTT TTTAATTGAA TATTTAAGAT TATAACATAT ATTTAAAGTG

201 TATCTAGATA CTTTTTGGGA ATGTTGGATA AAGGAGATAA AAAATGTATA

251 AGAGATTATT TATTTCACAT GTAATTTTGA TATTCGCACT GATATTAGTT

301 ATTTCTACAC CCAACGTTTT AGCAGAGAGT CAACCAGATC CTAAACCAGA

351 TGAGTTGCAC AAATCGAGTA AATTCACTGG TTTGATGGAA GATATGAAAG

401 TTTTGTATGA TGATAATCAT GTATCAGCAA TAAACGTTAA ATCTATAGAT

451 CAATTTCTAT ACTTTGACTT AATATATTCT ATTAAGGACA CTAAGTTAGG

501 GGATTATGAT AATGTTCGAG TCGAATTTAA AAACAAAGAT TTAGCTGATA

551 AATACAAAGA TAAATACGTA GATGTGTTTG GAGCTAATTA TTATTATCAA

601 TGTTATTTTT CTAAAAAAAC GAATGATATT AATTCGCATC AAACTGACAA

651 ACGAAAAACT TGTATGTATG GTGGTGTAAC TGAGCATAAT GGAAACCAAT

701 TAGATAAATA TAGAAGTATT ACTGTTCGGG TATTTGAAGA TGGTAAAAAT

751 TTATTATCTT TTGACGTACA AACTAATAAG AAAAAGGTGA CTGCTCAAGA

801 ATTAGATTAC CTAACTCGTC ACTATTTGGT GAAAAATAAA AAACTCTATG

851 AATTTAACAA CTCGCCTTAT GAAACGGGAT ATATTAAATT TATAGAAAAT

901 GAGAATAGCT TTTGGTATGA CATGATGCCT GCACCAGGAG ATAAATTTGC

951 CCAATCTAAA TATTTAATGA TGTACAATGA CAATAAAATG GTTGATTCTA

1001 AAGATGTGAA GATTGAAGTT TATCTTACGA CAAAGAAAAA GTGAAATTAT

1051 ATTTTAGAAA AGTAAATATG AAGAGTTAGT AATTAAGGCA GGCACTTATA

1101 GAGTACCTGC CTTTTCTAAT ATTATTTAGT TATAGTTATT TTTGTTATAT

1151 CTCTCTGATT TAGCATTAAC CCCTTGTTGC CATTATAGTT TTCACCAACT

1201 TTAGCTGAAA TTGGGGGATC ATTTTTATCT TTACTATGGA TAGTTACTGT

1251 GTCGCCGTTT TTAACGATTT GTTTCTCTTT TAATTTGTCA GTTAATTTTT

1301 TCCATGCATC ATTTGCGTCA AACCTATTTC CATTTGGATT TATTCTTGAC

1351 AAATCAATTC TTTTAACACT ATCGGTATTA ATCGGCTTGT TATTAAAATT

1401 ACTAAGTTCA TCTAAATCAG CTGTACCCGT AATACTACTT TCGCCACCAT

1451 TATTTAAATT GTACGTAACA CCAACTGTCT CATTTGCTGT TTTATCGATA

1501 ATATTTGCTT CTTTCAAAGC ATCTCTTACA TTTTTCCATA AGTCTCTATC

1551 TGTTATTTCA GAAGCCTTTG CAACGTTATT AATACCATTA TAATTTGAAG

1601 AAGAATGAAA ACCTGAACCT ACTGTTGTTA AAACTAAAGC ACTTGCTATC

1651 AATGTTCTTG TTAATAGTTT TTTATTCATT TTATTTTCTC CTATAACTTA

1701 TTTGCAATCG AT

SEQ ID NO:6
Staphylococcal enterotoxin B vaccine, B2360210
Amino acid sequence:

MYKRLFISHVILIFALILVISTPNVLAESQPDPKPDELHKSSKF

TGLMEDMKVLYDDNHVSAINVKSIDQFLYFDLIYSIKDTKLGDYDNVRVEFKNKDLAD

KYKDKYVDVFGANYYYQCYFSKKTNDINSHQTDKRKTCMYGGVTEHNGNQLDKYRSIT

VRVFEDGKNLLSFDVQTNKKKVTAQELDYLTRHYLVKNKKLYEFNNSPYETGYIKFIE

NENSFWYDMMPAPGDKFAQSKYLMMYNDNKMVDSKDVKIEVYLTTKKK

SEQ ID NO: 7
Staphylococcal enterotoxin B vaccine, B899445P, periplasmic
Gene sequence:

1 GAACTAGGTA GAAAAATAAT TATGAGAAAA CACTATGTTG TTAAAGATGT
51 TTTCGTATAT AAGTTTAGGT GATGTATAGT TACTTAATTT TAAAAGCATA
101 ACTTAATTAA TATAAATAAC ATGAGATTAT TAAATATAAT TAAGTTTCTT
151 TTAATGTTTT TTTAATTGAA TATTTAAGAT TATAACATAT ATTTAAAGTG
201 TATCTAGATA CTTTTTGGGA ATGTTGGATA AAGGAGATAA AAAATGTATA
251 AGAGATTATT TATTTCACAT GTAATTTTGA TATTCGCACT GATATTAGTT
301 ATTTCTACAC CCAACGTTTT AGCAGAGAGT CAACCAGATC CTAAACCAGA
351 TGAGTTGCAC AAATCGAGTA AATTCACTGG TTTGATGGAA AATATGAAAG
401 TTTTGTATGA TGATAATCAT GTATCAGCAA TAAACGTTAA ATCTATAGAT
451 CAATTTCGAT ACTTTGACTT AATATATTCT ATTAAGGACA CTAAGTTAGG
501 GAATTATGAT AATGTTCGAG TCGAATTTAA AAACAAAGAT TTAGCTGATA
551 AATACAAAGA TAAATACGTA GATGTGTTTG GAGCTAATGC TTATTATCAA
601 TGTGCTTTTT CTAAAAAAAC GAATGATATT AATTCGCATC AAACTGACAA
651 ACGAAAAACT TGTATGTATG GTGGTGTAAC TGAGCATAAT GGAAACCAAT
701 TAGATAAATA TAGAAGTATT ACTGTTCGGG TATTTGAAGA TGGTAAAAAT
751 TTATTATCTT TTGACGTACA AACTAATAAG AAAAAGGTGA CTGCTCAAGA
801 ATTAGATTAC CTAACTCGTC ACTATTTGGT GAAAAATAAA AAACTCTATG
851 AATTTAACAA CTCGCCTTAT GAAACGGGAT ATATTAAATT TATAGAAAAT
901 GAGAATAGCT TTTGGTATGA CATGATGCCT GCACCAGGAG ATAAATTTGA
951 CCAATCTAAA TATTTAATGA TGTACAATGA CAATAAAATG GTTGATTCTA
1001 AAGATGTGAA GATTGAAGTT TATCTTACGA CAAAGAAAAA GTGAAATTAT
1051 ATTTTAGAAA AGTAAATATG AAGAGTTAGT AATTAAGGCA GGCACTTATA
1101 GAGTACCTGC CTTTTCTAAT ATTATTTAGT TATAGTTATT TTTGTTATAT
1151 CTCTCTGATT TAGCATTAAC CCCTTGTTGC CATTATAGTT TTCACCAACT
1201 TTAGCTGAAA TTGGGGGATC ATTTTTATCT TTACTATGGA TAGTTACTGT
1251 GTCGCCGTTT TTAACGATTT GTTTCTCTTT TAATTTGTCA GTTAATTTTT

1301 TCCATGCATC ATTTGCGTCA AACCTATTTC CATTTGGATT TATTCTTGAC

1351 AAATCAATTC TTTTAACACT ATCGGTATTA ATCGGCTTGT TATTAAAATT

1401 ACTAAGTTCA TCTAAATCAG CTGTACCCGT AATACTACTT TCGCCACCAT

1451 TATTTAAATT GTACGTAACA CCAACTGTCT CATTTGCTGT TTTATCGATA

1501 ATATTTGCTT CTTTCAAAGC ATCTCTTACA TTTTTCCATA AGTCTCTATC

1551 TGTTATTTCA GAAGCCTTTG CAACGTTATT AATACCATTA TAATTTGAAG

1601 AAGAATGAAA ACCTGAACCT ACTGTTGTTA AAACTAAAGC ACTTGCTATC

1651 AATGTTCTTG TTAATAGTTT TTTATTCATT TTATTTTCTC CTATAACTTA

1701 TTTGCAATCG AT

SEQ ID NO: 8
Staphylococcal enterotoxin B vaccine, B899445P, periplasmic
Amino acid sequence:

MYKRLFISHVILIFALILVISTPNVLAESQPDPKPDELHKSSKF

TGLMENMKVLYDDNHVSAINVKSIDQFRYFDLIYSIKDTKLGNYDNVRVEFKNKDLAD

KYKDKYVDVFGANAYYQCAFSKKTNDINSHQTDKRKTCMYGGVTEHNGNQLDKYRSIT

VRVFEDGKNLLSFDVQTNKKKVTAQELDYLTRHYLVKNKKLYEFNNSPYETGYIKFIE

NENSFWYDMMPAPGDKFDQSKYLMMYNDNKMVDSKDVKIEVYLTTKKK

SEQ ID NO:9
Staphylococcal enterotoxin B vaccine, B899445C, cytoplasmic
Gene sequence:

325 ATGAGT CAACCAGATC CTAAACCAGA

351 TGAGTTGCAC AAATCGAGTA AATTCACTGG TTTGATGGAA AATATGAAAG

401 TTTTGTATGA TGATAATCAT GTATCAGCAA TAAACGTTAA ATCTATAGAT

451 CAATTTCGAT ACTTTGACTT AATATATTCT ATTAAGGACA CTAAGTTAGG

501 GAATTATGAT AATGTTCGAG TCGAATTTAA AAACAAAGAT TTAGCTGATA

551 AATACAAAGA TAAATACGTA GATGTGTTTG GAGCTAATGC TTATTATCAA

601 TGTGCTTTTT CTAAAAAAAC GAATGATATT AATTCGCATC AAACTGACAA

651 ACGAAAAACT TGTATGTATG GTGGTGTAAC TGAGCATAAT GGAAACCAAT
701 TAGATAAATA TAGAAGTATT ACTGTTCGGG TATTTGAAGA TGGTAAAAAT
751 TTATTATCTT TTGACGTACA AACTAATAAG AAAAAGGTGA CTGCTCAAGA
801 ATTAGATTAC CTAACTCGTC ACTATTTGGT GAAAAATAAA AAACTCTATG
851 AATTTAACAA CTCGCCTTAT GAAACGGGAT ATATTAAATT TATAGAAAAT
901 GAGAATAGCT TTTGGTATGA CATGATGCCT GCACCAGGAG ATAAATTTGA
951 CCAATCTAAA TATTTAATGA TGTACAATGA CAATAAAATG GTTGATTCTA
1001 AAGATGTGAA GATTGAAGTT TATCTTACGA CAAAGAAAAA GTGAAATTAT
1051 ATTTTAGAAA AGTAAATATG AAGAGTTAGT AATTAAGGCA GGCACTTATA
1101 GAGTACCTGC CTTTTCTAAT ATTATTTAGT TATAGTTATT TTTGTTATAT
1151 CTCTCTGATT TAGCATTAAC CCCTTGTTGC CATTATAGTT TTCACCAACT
1201 TTAGCTGAAA TTGGGGGATC ATTTTTATCT TTACTATGGA TAGTTACTGT
1251 GTCGCCGTTT TTAACGATTT GTTTCTCTTT TAATTTGTCA GTTAATTTTT
1301 TCCATGCATC ATTTGCGTCA AACCTATTTC CATTTGGATT TATTCTTGAC
1351 AAATCAATTC TTTTAACACT ATCGGTATTA ATCGGCTTGT TATTAAAATT
1401 ACTAAGTTCA TCTAAATCAG CTGTACCCGT AATACTACTT TCGCCACCAT
1451 TATTTAAATT GTACGTAACA CCAACTGTCT CATTTGCTGT TTTATCGATA
1501 ATATTTGCTT CTTTCAAAGC ATCTCTTACA TTTTTCCATA AGTCTCTATC
1551 TGTTATTTCA GAAGCCTTTG CAACGTTATT AATACCATTA TAATTTGAAG
1601 AAGAATGAAA ACCTGAACCT ACTGTTGTTA AAACTAAAGC ACTTGCTATC
1651 AATGTTCTTG TTAATAGTTT TTTATTCATT TTATTTTCTC CTATAACTTA
1701 TTTGCAATCG AT

SEQ ID NO:10
Staphylococcal enterotoxin B vaccine, B899445C, cytoplasmic
Amino acid sequence:

MSQPDPKPDELHKSSKFTGLMENMKVLYDDNHVSAINVKSIDQFRYFDLIYSIKDTKL

GNYDNVRVEFKNKDLADKYKDKYVDVFGANAYYQCAFSKKTNDINSHQTDKRKTCMYG

GVTEHNGNQLDKYRSITVRVFEDGKNLLSFDVQTNKKKVTAQELDYLTRHYLVKNKKL

YEFNNSPYETGYIKFIENENSFWYDMMPAPGDKFDQSKYLMMYNDNKMVDSKDVKIEV

YLTTKKK

SEQ ID NO: 11
Staphylococcal toxic shock syndrome toxin-1 vaccine TST30
Gene sequence:

1 TAAGGAGAAT TAAAAATGAA TAAAAAATTA CTAATGAATT TTTTTATCGT

51 AAGCCCTTTG TTGCTTGCGA CAACTGCTAC AGATTTTACC CCTGTTCCCT

101 TATCATCTAA TCAAATAATC AAAACTGCAA AAGCATCTAC AAACGATAAT

151 ATAAAGGATT TGCTAGACTG GTATAGTAGT GGGTCTGACA CTTTTACAAA

201 TAGTGAAGTT TTAGATAATT CCAGAGGATC TATGCGTATA AAAAACACAG

251 ATGGCAGCAT CAGCTTGATA ATTTTTCCGA GTCCTTATTA TAGCCCTGCT

301 TTTACAAAAG GGGAAAAAGT TGACTTAAAC ACAAAAAGAA CTAAAAAAAG

351 CCAACATACT AGCGAAGGAA CTTATATCCA TTTCCAAATA AGTGGCGTTA

401 CAAATACTGA AAAATTACCT ACTCCAATAG AACTACCTTT AAAAGTTAAG

451 GTTCATGGTA AAGATAGCCC CTTAAAGTAT GGGCCAAAGT TCGATAAAAA

501 ACAATTAGCT ATATCAACTT TAGACTTTGA AATTCGTCAT CAGCTAACTC

551 AAATACATGG ATTATATCGT TCAAGCGATA AAACGGGTGG TTATTGGAAA

601 ATAACAATGA ATGACGGATC CACATATCAA AGTGATTTAT CTAAAAAGTT

651 TGAATACAAT ACTGAAAAAC CACCTATAAA TATTGATGAA ATAAAAACTA

701 TAGAAGCAGA AATTAATTAA TTTACCACTT T

SEQ ID NO: 12
Staphylococcal toxic shock syndrome toxin-1 vaccine TST30
Amino acid sequence:

MNKKLLMNFFIVSPLLLATTATDFTPVPLSSNQIIKTAKASTND

NIKDLLDWYSSGSDTFTNSEVLDNSRGSMRIKNTDGSISLIIFPSPYYSPAFTKGEKV

DLNTKRTKKSQHTSEGTYIHFQISGVTNTEKLPTPIELPLKVKVHGKDSPLKYGPKFD

KKQLAISTLDFEIRHQLTQIHGLYRSSDKTGGYWKITMNDGSTYQSDLSKKFEYNTEK

PPINIDEIKTIEAEIN..

SEQ ID NO: 13
Staphylococcus enterotoxin C1 vaccine SEC45
gene sequence

1 ATCATTAAAT ATAATTAATT TTCTTTTAAT ATTTTTTTAA TTGAATATTT

51 AAGATTATAA GATATATTTA AAGTGTATCT AGATACTTTT TGGGAATGTT

101 GGATGAAGGA GATAAAAATG AATAAGAGTC GATTTATTTC ATGCGTAATT

151 TTGATATTCG CACTTATACT AGTTCTTTTT ACACCCAACG TATTAGCAGA

201 GAGCCAACCA GACCCTACGC CAGATGAGTT GCACAAAGCG AGTAAATTCA

251 CTGGTTTGAT GGAAAATATG AAAGTTTTAT ATGATGATCA TTATGTATCA

301 GCAACTAAAG TTAAGTCTGT AGATAAATTT AGGGCACATG ATTTAATTTA

351 TAACATTAGT GATAAAAAAC TGAAAAATTA TGACAAAGTG AAAACAGAGT

401 TATTAAATGA AGGTTTAGCA AAGAAGTACA AAGATGAAGT AGTTGATGTG

451 TATGGATCAA ATTACTATGT AAACTGCTAT TTTTCATCCA AAGATAATGT

501 AGGTAAAGTT ACAGGTGGCA AAACTTGTAT GTATGGAGGA ATAACAAAAC

551 ATGAAGGAAA CCACTTTGAT AATGGGAACT TACAAAATGT ACTTATAAGA

601 GTTTATGAAA ATAAAAGAAA CACAATTTCT TTTGAAGTGC AAACTGATAA

651 GAAAAGTGTA ACAGCTCAAG AACTAGACAT AAAAGCTAGG AATTTTTTAA

701 TTAATAAAAA AAATTTGTAT GAGTTTAACA GTTCACCATA TGAAACAGGA

751 TATATAAAAT TTATTGAAAA TAACGGCAAT ACTTTTTGGT ATGATATGAT

801 GCCTGCACCA GGCGATAAGT TTGACCAATC TAAATATTTA ATGATGTACA

851 ACGACAATAA AACGGTTGAT TCTAAAAGTG TGAAGATAGA AGTCCACCTT

901 ACAACAAAGA ATGGATAATG TTAATCCGAT TTTGATATAA AAAGTGAAAG

951 TATTAGATAT ATTTGAAAGG TAAGTACTTC GGTGCTTGCC TTTTTAGGAT

1001 GCATATATAT AGATTAAACC GCACTTCTAT ATTAATAGAA AGTGCGGTTA

1051 TTTATACACT CAATCTAAAC TATAATAATT GGAATCATCT TCAAA

SEQ ID NO: 14
Staphylococcus enterotoxin C1 vaccine SEC45
Amino acid sequence:

MNKSRFISCVILIFALILVLFTPNVLAESQPDPTPDELHKASKF

TGLMENMKVLYDDHYVSATKVKSVDKFRAHDLIYNISDKKLKNYDKVKTELLNEGLAK

KYKDEVVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLI

RVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINKKNLYEFNSSPYETGYIKFIEN

NGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVHLTTKNG"

SEQ ID NO:15
Streptococcal pyrogenic exotoxin A vaccine SPEA42
Gene sequence:

1 TCATGTTTGA CAGCTTATCA TCGATAAGCT TACTTTTCGA ATCAGGTCTA

51 TCCTTGAAAC AGGTGCAACA TAGATTAGGG CATGGAGATT TACCAGACAA

101 CTATGAACGT ATATACTCAC ATCACGCAAT CGGCAATTGA TGACATTGGA

151 ACTAAATTCA ATCAATTTGT TACTAACAAG CAACTAGATT GACAACTAAT

201 TCTCAACAAA CGTTAATTTA ACAACATTCA AGTAACTCCC ACCAGCTCCA

251 TCAATGCTTA CCGTAAGTAA TCATAACTTA CTAAAACCTT GTTACATCAA

301 GGTTTTTTCT TTTTGTCTTG TTCATGAGTT ACCATAACTT TCTATATTAT

351 TGACAACTAA ATTGACAACT CTTCAATTAT TTTTCTGTCT ACTCAAAGTT

401 TTCTTCATTT GATATAGTCT AATTCCACCA TCACTTCTTC CACTCTCTCT

451 ACCGTCACAA CTTCATCATC TCTCACTTTT TCGTGTGGTA ACACATAATC

501 AAATATCTTT CCGTTTTTAC GCACTATCGC TACTGTGTCA CCTAAAATAT

551 ACCCCTTATC AATCGCTTCT TTAAACTCAT CTATATATAA CATATTTCAT

601 CCTCCTACCT ATCTATTCGT AAAAAGATAA AAATAACTAT TGTTTTTTTT

651 GTTATTTTAT AATAAAATTA TTAATATAAG TTAATGTTTT TTAAAAATAT

701 ACAATTTTAT TCTATTTATA GTTAGCTATT TTTTCATTGT TAGTAATATT

751 GGTGAATTGT AATAACCTTT TTAAATCTAG AGGAGAACCC AGATATAAAA

801 TGGAGGAATA TTAATGGAAA ACAATAAAAA AGTATTGAAG AAAATGGTAT

851 TTTTTGTTTT AGTGACATTT CTTGGACTAA CAATCTCGCA AGAGGTATTT

901 GCTCAACAAG ACCCCGATCC AAGCCAACTT CACAGATCTA GTTTAGTTAA

951 AAACCTTCAA AATATATATT TTCTTTATGA GGGTGACCCT GTTACTCACG

1001 AGAATGTGAA ATCTGTTGAT CAACTTAGAT CTCACGATTT AATATATAAT

1051 GTTTCAGGGC CAAATTATGA TAAATTAAAA ACTGAACTTA AGAACCAAGA

1101 GATGGCAACT TTATTTAAGG ATAAAAACGT TGATATTTAT GGTGTAGAAT

1151 ATTACCATCT CTGTTATTTA TGTGAAAATG CAGAAAGGAG TGCATGTATC

1201 TACGGAGGGG TAACAAATCA TGAAGGGAAT CATTTAGAAA TTCCTAAAAA

1251 GATAGTCGTT AAAGTATCAA TCGATGGTAT CCAAAGCCTA TCATTTGATA

1301 TTGAAACAAA TAAAAAAATG GTAACTGCTC AAGAATTAGA CTATAAAGTT

1351 AGAAAATATC TTACAGATAA TAAGCAACTA TATACTAATG GACCTTCTAA

1401 ATATGAAACT GGATATATAA AGTTCATACC TAAGAATAAA GAAAGTTTTT

1451 GGTTTGATTT TTTCCCTGAA CCAGAATTTA CTCAATCTAA ATATCTTATG

1501 ATATATAAAG ATAATGAAAC GCTTGACTCA AACACAAGCC AAATTGAAGT

1551 CTACCTAACA ACCAAGTAAC TTTTTGCTTT TGGCAACCTT ACCTACTGCT

1601 GGATTTAGAA ATTTTATTGC AATTCTTTTA TTAATGTAAA AACCGCTCAT

1651 TTGATGAGCG GTTTTGTCTT ATCTAAAGGA GCTTTACCTC CTAATGCTGC

1701 AAAATTTTAA ATGTTGGATT TTTGTATTTG TCTATTGTAT TTGATGGGTA

1751 ATCCCATTTT TCGACAGACA TCGTCGTGCC ACCTCTAACA CCAAAATCAT

1801 AGACAGGAGC TTGTAGCTTA GCAACTATTT TATCGTC

SEQ ID NO:16
Streptococcal pyrogenic exotoxin A vaccine SPEA42
Amino acid sequence:

MENNKKVLKKMVFFVLVTFLGLTISQEVFAQQDPDPSQLHRSSL

VKNLQNIYFLYEGDPVTHENVKSVDQLRSHDLIYNVSGPNYDKLKTELKNQEMATLFK

DKNVDIYGVEYYHLCYLCENAERSACIYGGVTNHEGNHLEIPKKIVVKVSIDGIQSLS

FDIETNKKMVTAQELDYKVRKYLTDNKQLYTNGPSKYETGYIKFIPKNKESFWFDFFP

EPEFTQSKYLMIYKDNETLDSNTSQIEVYLTTK"

What is claimed is:

1. An isolated and purified superantigen toxin DNA fragment which has been altered such that binding of the encoded altered toxin to either the MHC class II or T cell antigen receptor is altered.

2. An isolated and purified DNA fragment according to claim 1, wherein said superantigen toxin is Staphylococcal enterotoxin A having the sequence of SEQ ID NO:1 or a portion thereof, or an allelic portion thereof.

3. An isolated and purified DNA fragment according to claim 1, wherein said superantigen toxin is Staphylococcal enterotoxin A having the sequence of SEQ ID NO:3 or a portion thereof, or an allelic portion thereof.

4. An isolated and purified DNA fragment according to claim 1, wherein said superantigen toxin is Staphylococcal enterotoxin B having the sequence of SEQ ID NO:5 or a portion thereof, or an allelic portion thereof.

5. An isolated and purified DNA fragment according to claim 1, wherein said superantigen toxin is Staphylococcal enterotoxin B having the sequence of SEQ ID NO:7 or a portion thereof, or an allelic portion thereof.

6. An isolated and purified DNA fragment according to claim 1, wherein said superantigen toxin is Staphylococcal enterotoxin B having the sequence of SEQ ID NO:9 or a portion thereof, or an allelic portion thereof.

7. An isolated and purified DNA fragment according to claim 1, wherein said superantigen toxin is toxic shock syndrome toxin-1 having the sequence of SEQ ID NO:11 or a portion thereof, or an allelic portion thereof.

8. An isolated and purified DNA fragment according to claim 1, wherein said superantigen toxin is Staphylococcal enterotoxin C1 having the sequence of SEQ ID NO:13 or a portion thereof, or an allelic portion thereof.

9. An isolated and purified DNA fragment according to claim 1, wherein said superantigen toxin is Streptococcal pyrogenic exotoxin A having the sequence of SEQ ID NO:15 or a portion thereof, or an allelic portion thereof.

10. An isolated and purified DNA fragment according to claim 2, wherein said fragment encodes the amino acid sequence of SEQ ID NO:2 or a portion thereof, or an allelic portion thereof.

11. An isolated and purified DNA fragment according to claim 3, wherein said fragment encodes the amino acid sequence of SEQ ID NO:4 or a portion thereof, or an allelic portion thereof.

12. An isolated and purified DNA fragment according to claim 4, wherein said fragment encodes the amino acid sequence of SEQ ID NO:6 or a portion thereof, or an allelic portion thereof.

13. An isolated and purified DNA fragment according to claim 5, wherein said fragment encodes the amino acid sequence of SEQ ID NO:8 or a portion thereof, or an allelic portion thereof.

14. An isolated and purified DNA fragment according to claim 6, wherein said fragment encodes the amino acid sequence of SEQ ID NO:10 or a portion thereof, or an allelic portion thereof.

15. An isolated and purified DNA fragment according to claim 7, wherein said fragment encodes the amino acid sequence of SEQ ID NO:12 or a portion thereof, or an allelic portion thereof.

16. An isolated and purified DNA fragment according to claim 8, wherein said fragment encodes the amino acid sequence of SEQ ID NO:14 or a portion thereof, or an allelic portion thereof.

17. An isolated and purified DNA fragment according to claim 9, wherein said fragment encodes the amino acid sequence of SEQ ID NO:16 or a portion thereof, or an allelic portion thereof.

18. A recombinant DNA construct comprising:

(i) a vector, and

(ii) an isolated and purified altered superantigen toxin DNA fragment according to claim 1.

19. A recombinant DNA construct according to claim 18, wherein said DNA fragment has the sequence according to SEQ ID NO:1 or a portion thereof, or an allelic portion thereof.

20. A recombinant DNA construct according to claim 18, wherein said DNA fragment has the sequence according to SEQ ID NO:3 or a portion thereof, or an allelic portion thereof.

21. A recombinant DNA construct according to claim 18, wherein said DNA fragment has the sequence according to SEQ ID NO:5 or a portion thereof, or an allelic portion thereof.

22. A recombinant DNA construct according to claim 18, wherein said DNA fragment has the sequence according to SEQ ID NO:7 or a portion thereof, or an allelic portion thereof.

23. A recombinant DNA construct according to claim 18, wherein said DNA fragment has the sequence according to SEQ ID NO:9 or a portion thereof, or an allelic portion thereof.

24. A recombinant DNA construct according to claim 18, wherein said DNA fragment has the sequence according to SEQ ID NO:11 or a portion thereof, or an allelic portion thereof.

25. A recombinant DNA construct according to claim 18, wherein said DNA fragment has the sequence according to SEQ ID NO:13 or a portion thereof, or an allelic portion thereof.

26. A recombinant DNA construct according to claim 18, wherein said DNA fragment has the sequence according to SEQ ID NO:15 or a portion thereof, or an allelic portion thereof.

27. The recombinant DNA construct according to claim 19, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO:2.

28. The recombinant DNA construct according to claim 20, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO:4.

29. The recombinant DNA construct according to claim 21, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO:6.

30. The recombinant DNA construct according to claim 22, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO:8.

31. The recombinant DNA construct according to claim 23, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO:10.

32. The recombinant DNA construct according to claim 24, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO:12.

33. The recombinant DNA construct according to claim 25, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO:14.

34. The recombinant DNA construct according to claim 26, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO:16.

35. A recombinant DNA construct according to claim 19 wherein said construct is pETA489270P.

36. A recombinant DNA construct according to claim 20 wherein said construct is pETA489270C.

37. A recombinant DNA construct according to claim 21 wherein said construct is pETB2360210.

38. A recombinant DNA construct according to claim 22 wherein said construct pETB899445P.

39. A recombinant DNA construct according to claim 23 wherein said construct is pETB899445C.

40. A recombinant DNA construct according to claim 24 wherein said construct is pETTST30.

41. A recombinant DNA construct according to claim 25 wherein said construct is pETSEC45.

42. A recombinant DNA construct according to claim 26 wherein said construct is pETSPEA42.

43. A recombinant DNA construct according to claim 18, wherein said vector is an expression vector.

44. A host cell transformed with a recombinant DNA construct according to claim 18.

45. A host cell transformed with a recombinant DNA construct according to claim 27.

46. A host cell transformed with a recombinant DNA construct according to claim 28.

47. A host cell transformed with a recombinant DNA construct according to claim 29.

48. A host cell transformed with a recombinant DNA construct according to claim 30.

49. A host cell transformed with a recombinant DNA construct according to claim 31.

50. A host cell transformed with a recombinant DNA construct according to claim 32.

51. A host cell transformed with a recombinant DNA construct according to claim 33.

52. A host cell transformed with a recombinant DNA construct according to claim 34.

53. A host cell according to claim 44, wherein said cell is prokaryotic.

54. A host cell according to claim 45, wherein said cell is prokaryotic.

55. A host cell according to claim 46, wherein said cell is prokaryotic.

56. A host cell according to claim 47, wherein said cell is prokaryotic.

57. A host cell according to claim 48, wherein said cell is prokaryotic.

58. A host cell according to claim 49, wherein said cell is prokaryotic.

59. A host cell according to claim 50, wherein said cell is prokaryotic.

60. A host cell according to claim 51, wherein said cell is prokaryotic.

61. A host cell according to claim 52, wherein said cell is prokaryotic.

62. A method for producing altered superantigen toxin comprising culturing the cells according to claim 44, under conditions such that said DNA fragment is expressed and said superantigen toxin is thereby produced, and isolating said superantigen toxin.

63. A method for producing altered superantigen toxin comprising culturing the cells according to claim 45, under conditions such that said DNA fragment is expressed and said superantigen toxin is thereby produced, and isolating said superantigen toxin.

64. A method for producing altered superantigen toxin comprising culturing the cells according to claim 46, under conditions such that said DNA fragment is expressed and said superantigen toxin is thereby produced, and isolating said superantigen toxin.

65. A method for producing altered superantigen toxin comprising culturing the cells according to claim 47, under conditions such that said DNA fragment is expressed and said superantigen toxin

is thereby produced, and isolating said superantigen toxin.

66. A method for producing altered superantigen toxin comprising culturing the cells according to claim 48, under conditions such that said DNA fragment is expressed and said superantigen toxin is thereby produced, and isolating said superantigen toxin.

67. A method for producing altered superantigen toxin comprising culturing the cells according to claim 49, under conditions such that said DNA fragment is expressed and said superantigen toxin is thereby produced, and isolating said superantigen toxin.

68. A method for producing altered superantigen toxin comprising culturing the cells according to claim 50, under conditions such that said DNA fragment is expressed and said superantigen toxin is thereby produced, and isolating said superantigen toxin.

69. A method for producing altered superantigen toxin comprising culturing the cells according to claim 51, under conditions such that said DNA fragment is expressed and said superantigen toxin is thereby produced, and isolating said superantigen toxin.

70. A method for producing altered superantigen toxin comprising culturing the cells according to claim 52, under conditions such that said DNA fragment is expressed and said superantigen toxin

is thereby produced, and isolating said superantigen toxin.

71. An isolated and purified superantigen toxin which has been altered such that binding of the encoded altered toxin to either the MHC class II or T cell antigen receptor is altered.

72. An isolated and purified superantigen toxin according to claim 71 wherein said toxin is staphylococcal enterotoxin A.

73. An isolated and purified superantigen toxin according to claim 71 wherein said toxin is staphylococcal enterotoxin B.

74. An isolated and purified superantigen toxin according to claim 71 wherein said toxin is staphylococcal toxin shock syndrome toxin-1.

75. An isolated and purified superantigen toxin according to claim 71 wherein said toxin is staphylococcal enterotoxin C1.

76. An altered SEA superantigen toxin peptide according to claim 72 wherein position 92 has been changed to alanine.

77. An altered SEA superantigen toxin peptide according to claim 72 wherein position 70 has been changed to arginine.

78. An altered SEA superantigen toxin peptide according to claim 72 wherein position 48 has been changed to arginine.

79. An altered SEA superantigen toxin peptide according to claim 72 wherein position 64 has been mutated to alanine.

80. An altered SEB superantigen toxin peptide according to claim 73 wherein position 115 has been changed to alanine.

81. An altered SEB superantigen toxin peptide according to claim 73 wherein position 89 has been changed to alanine.

82. An altered SEB superantigen toxin peptide according to claim 73 wherein position 67 has been changed to glutamine.

83. An altered SEB superantigen toxin peptide according to claim 73 wherein position 94 has been changed to alanine.

84. An altered SEB superantigen toxin peptide according to claim 73 wherein position 61 has been changed to alanine.

85. A method for the diagnosis of superantigen-associated bacterial infection comprising the steps of:

(i) contacting a sample from an individual suspected of having a superantigen-associated bacterial infection with altered superantigen toxin; and

(ii) detecting the presence or absence of a superantigen-associated bacterial infection by detecting the presence or absence of a complex formed

between the altered superantigen toxin and antibodies specific therefor in the sample.

86. A method for the diagnosis of a superantigen toxin-associated bacterial infection according to claim 63 wherein the altered superantigen toxin is chosen from the group consisting of SPEa, SEB, SEA, TSST-1, SEC-1.

87. A superantigen toxin-associated infection diagnostic kit comprising an altered superantigen toxin according to claim 58 wherein said toxin is chosen from the group consisting of SPEa, SEB, SEA, TSST-1, and SEC-1, and ancillary reagents suitable for use in detecting the presence or absence of antibodies against superantigen toxin in a mammalian sample.

88. A vaccine comprising an altered superantigen toxin according to claim 58 effective for the production of antigenic and immunogenic response resulting in the protection of a mammal against superantigen-associated bacterial infection.

89. A vaccine according to claim 66 wherein said altered superantigen toxin is chosen from the group consisting of SPEa, SEB, SEA, TSST-1, and SEC-1.

90. A vaccine according to claim 67 wherein said vaccine further comprises at least one other different altered superantigen toxin chosen from the group consisting of SPEa, SEB, SEA, TSST-1, and SEC-1.

91. A vaccine according to claim 66, wherein the superantigen toxin is SEB and the vaccine is identified as B899445.

92. A vaccine according to claim 66, wherein the superantigen toxin is SEA and the vaccine is identified as A489270.

93. A bivalent vaccine according to claim 68 wherein said altered superantigen toxins are SEA and SEB.

94. A bivalent vaccine according to claim 71 wherein said toxin SEA is A489270 and SEB is B899445.

95. A multivalent vaccine against superantigen-associated bacterial infections comprising a combination of altered superantigen toxins selected from the group consisting essentially of TSST-1, SPEa, SEA, SEB, and SEC-1 or any portion or allelic form thereof, capable of eliciting protective antibodies against superantigen toxins in a pharmaceutically acceptable excipient in a pharmaceutically acceptable amount.

96. A therapeutic method for the treatment or amelioration of a superantigen-associated bacterial infection said method comprising administering to an individual in need of such treatment an effective amount of sera from individuals immunized with one of more altered superantigen toxin vaccine according to claim 67 in a pharmaceutically acceptable dose in a pharmaceutically acceptable excipient.

97. A therapeutic method for the treatment or amelioration of a superantigen-associated bacterial infection, said method comprising administering to an individual in need of such treatment an effective amount of antibodies against altered superantigen toxins in a pharmaceutically acceptable dose in a pharmaceutically acceptable excipient.

98. A therapeutic method for the treatment or amelioration of a superantigen-associated bacterial infection, said method comprising administering to an individual in need of such treatment an effective amount of altered superantigen toxins from streptococcal and staphylococcal bacteria in order to inhibit adhesionof superantigen bacterial toxin to MHC class II or T cell receptors by competitive inhibition of these interactions in a pharmaceutically acceptable dose in a pharmaceutically acceptable excipient.

99. A therapeutic method for the treatment of diseases that may not be associated directly with superantigen toxins by causing specific nonresponsiveness of T cell subsets or by expanding or stimulating specific T cell subsets, in vivo or ex vivo by use of altered superantigen toxin.

ABSTRACT

The present invention relates to genetically attenuated superantigen toxin vaccines altered such that superantigen attributes are absent, however the superantigen is effectively recognized and an appropriate immune response is produced. The attenuated superantigen toxins are shown to protect animals against challenge with wild type toxin. Methods of producing and using the altered superantigen toxins are described.

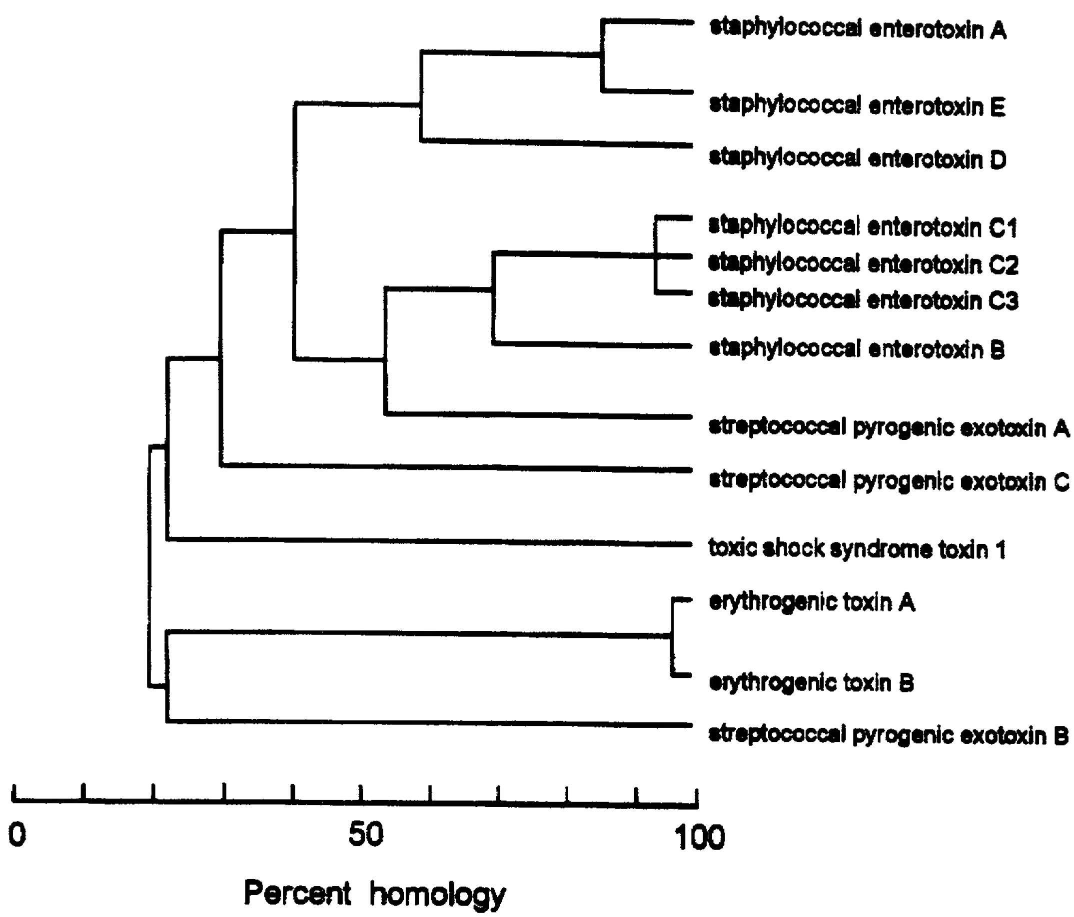
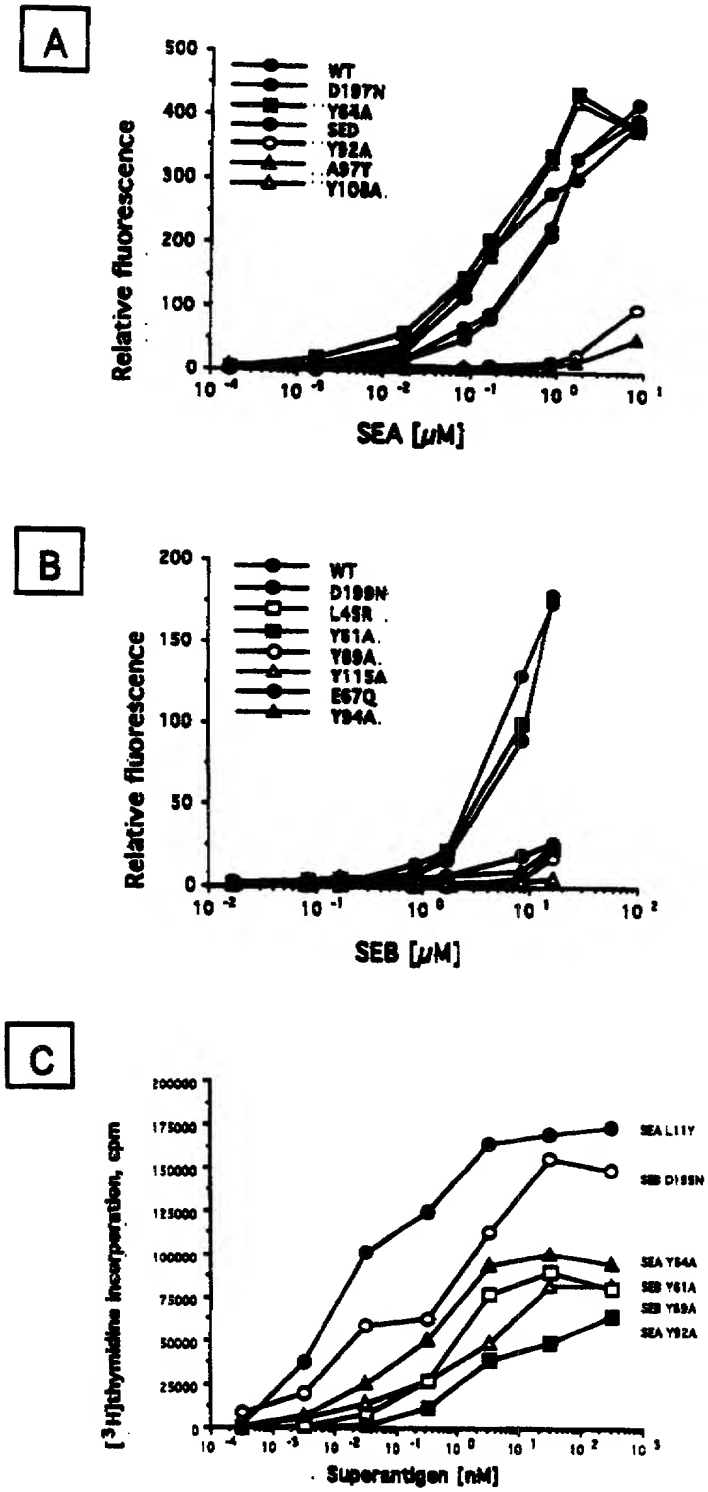


Fig 1 FIGURE 1


A
Relative fluorescence
SEA [μM]
WT
D197N
Y64A
SED
Y92A
A97Y
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B
Relative fluorescence
SEB [μM]
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D199N
L45R
Y61A
Y89A
Y115A
E67Q
Y94A
C
[3H]thymidine incorporation, cpm
Superantigen [nM]
SEA L11Y
SEB D199N
SEA Y64A
SEB Y61A
SEB Y89A
SEA Y92A

Fig. 2

```
            48                    70                    92                          108
SEA   ...SHDQF QHTILFKGFFTDHSWYNDLLV FDSKDIVDKYK.GKKVDLYGAY GYQCA...........GGTPNKTACM GGVTLHDNNRLTEEKK
SED   ...TGDQF ENTLLYKKFFTDLINFEDLLI FNSKEMAQHFK.SKNVDVYPIR SINCY...........GGEIDRTACT GGVTPHEGNKLKERKK
SEE   ...SDDQP ENTLLFKGFFTGHPWYNDLLV LGSKDATNKYK.GKKVDLYGAY GYQCA...........GGTPNKTACM GGVTLHDNNRLTEEKK
SEB   ...SIDQF YFDLIYSIKDTKLGNYDNVRV FKNKDLADKYK.DKYVDVFGAN YYQCYFSKKTNDINSHQTDKRKT.CM GGVTEHNGNQLD..KY
SEC1  ...SVDKF AHDLIYNISDKKLKNYDKVKT LLNEGLAKKYK.DEVVDVYGSN YVNCYFSSKDNVGKVTGG...KT.CM GGITKHEGNHFDNGNL
SEC2  ...SVDKF AHDLIYNISDKKLKNYDKVKT LLNEDLAKKYK.DEVVDVYGSN YVNCYFSSKDNVGKVTGG...KT.CM GGITKHEGNHFDNGNL
SEC3  ...SVDKF AHDLIYNISDKKLKNYDKVKT LLNEDLAKKYK.DEVVDVYGSN YVNCYFSSKDNVGKVTGG...KT.CM GGITKHEGNHFDNGNL
SPEa  ...SVDQL SHDLIYNVSG...PNYDKLKT LKNQEMATLFK.DKNVDIYGVE YHLCYLCENAE..........RSACI GGVTNHEGNHLEIPK.
TSST1 ...VLDNS GSMRIKNTD......GSISLI FPSPYYSPAFTKGEKVDLNTKR KKSQHTSEG..........TYIHF.Q SGVTNT EKLPT...P
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Fig. 3

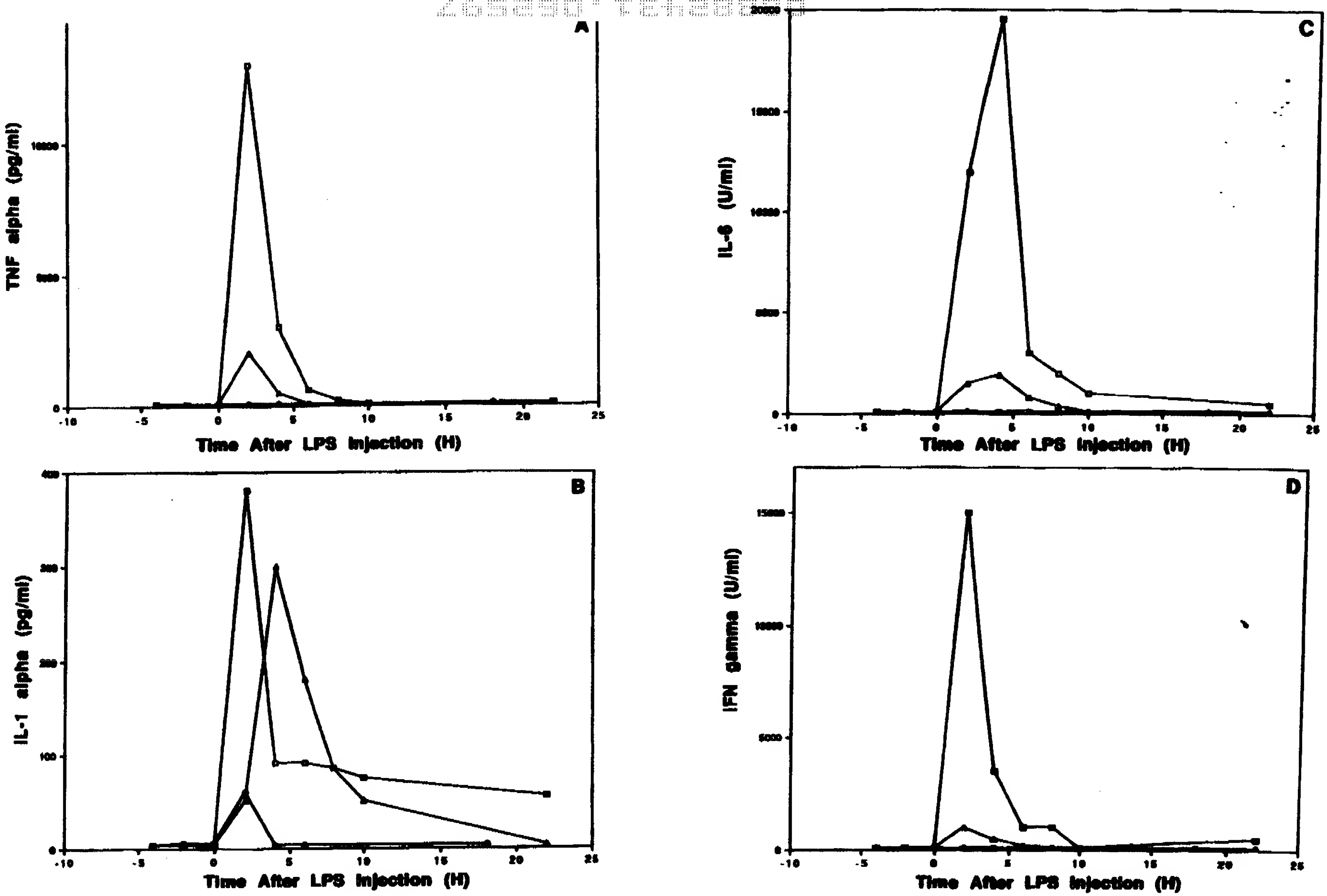

A
TNF alpha (pg/ml)
Time After LPS Injection (H)
B
IL-1 alpha (pg/ml)
Time After LPS Injection (H)
C
IL-6 (U/ml)
Time After LPS Injection (H)
D
IFN gamma (U/ml)
Time After LPS Injection (H)

Fig. 4

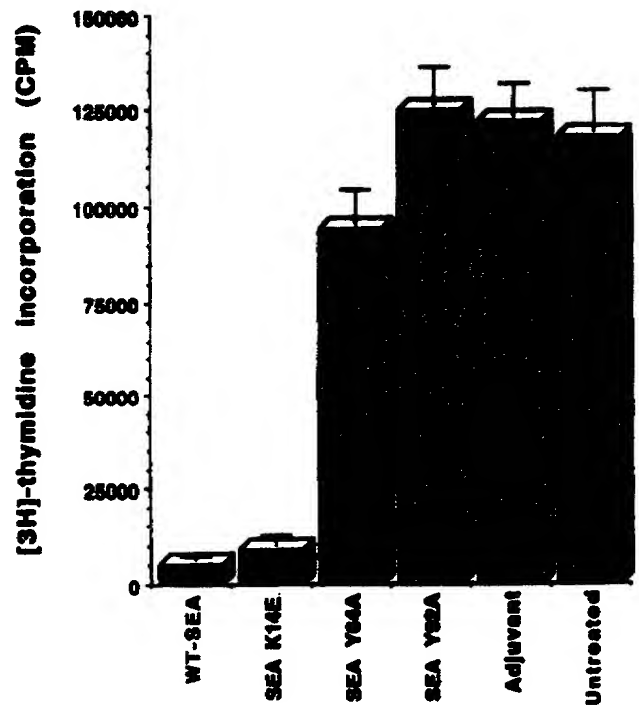

[3H]-thymidine incorporation (CPM)
150000
125000
100000
75000
50000
25000
0
WT-SEA
SEA K14E
SEA Y64A
SEA Y92A
Adjuvant
Untreated

Fig. 5

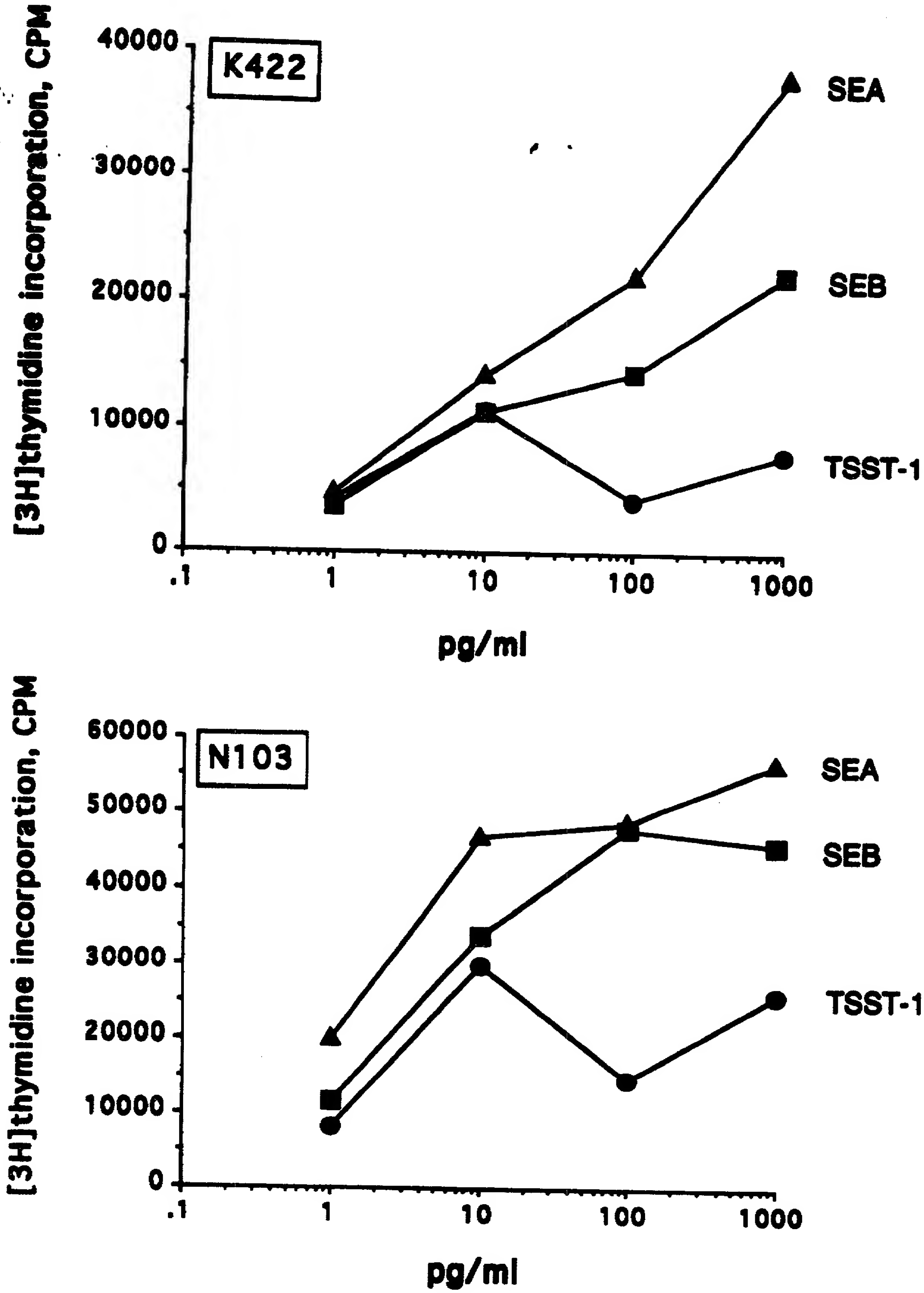
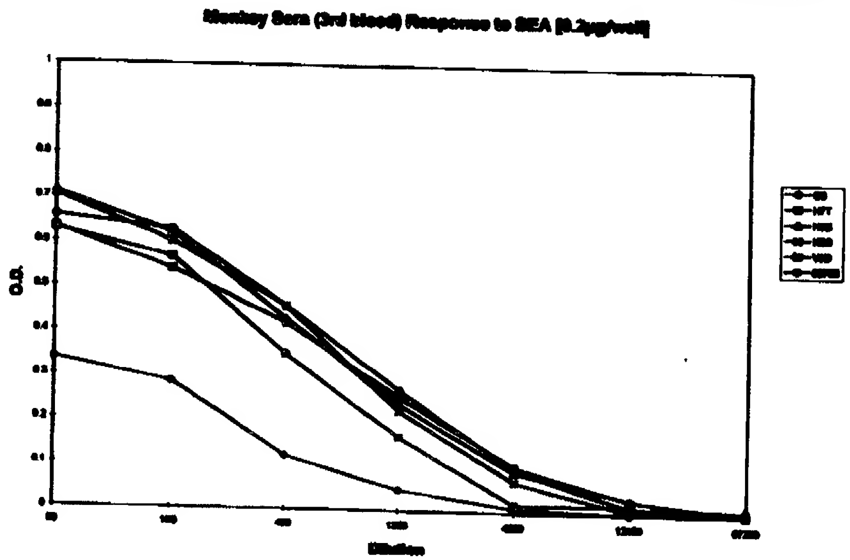
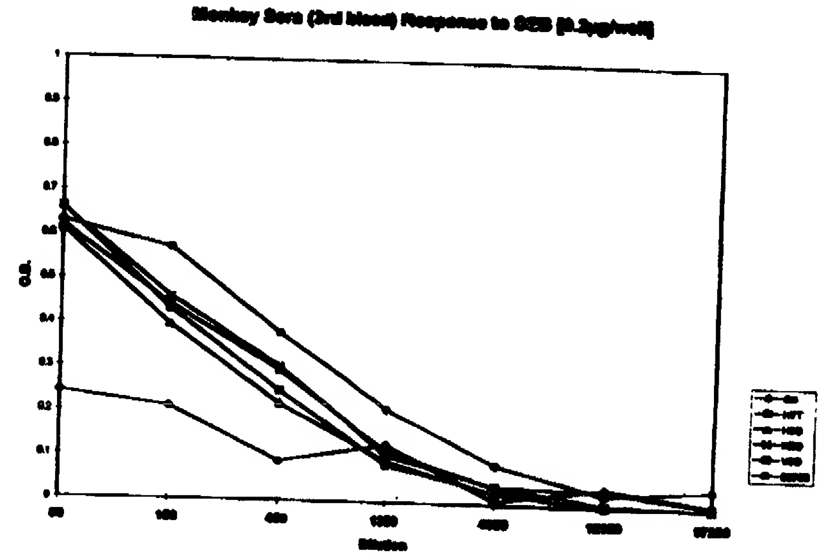

K422
[3H]thymidine incorporation, CPM
40000
30000
20000
10000
0
.1
1
10
100
1000
pg/ml
SEA
SEB
TSST-1
N103
[3H]thymidine incorporation, CPM
60000
50000
40000
30000
20000
10000
0
.1
1
10
100
1000
pg/ml
SEA
SEB
TSST-1

Fig. 6


Monkey Sera (3rd bleed) Response to SEA [0.2µg/well]
O.D.
Dilution


Monkey Sera (3rd bleed) Response to SEB [0.2µg/well]
O.D.
Dilution

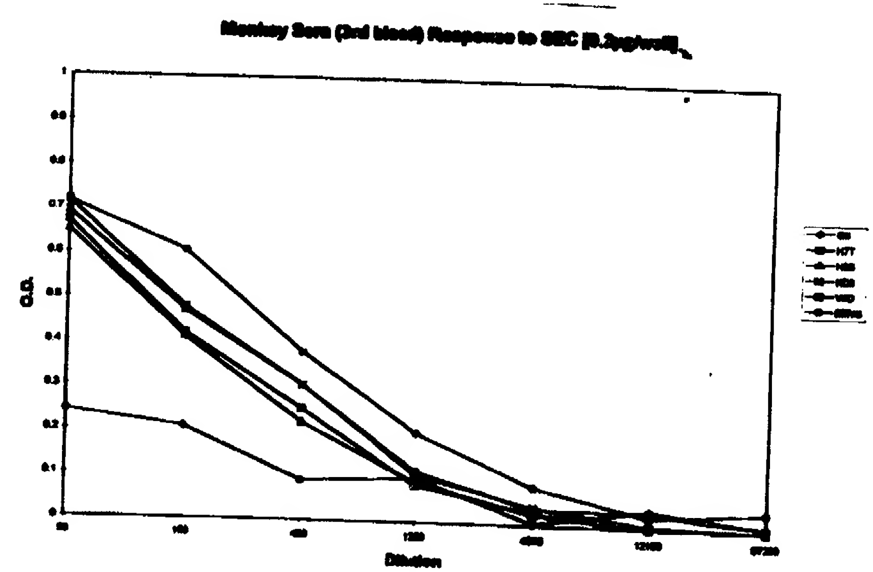

Monkey Sera (3rd bleed) Response to SEC [0.2µg/well]
O.D.
Dilution

Fig. 7

DECLARATION FOR PATENT APPLICATION IN THE UNITED STATES PATENT AND TRADEMARK OFFICE	[illegible]/SAP client/matter

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled BACTERIAL SUPERANTIGEN VACCINES.

the specification of which

[X] is attached hereto

☐ was filed on ______________________ as U.S. Application No. ______________________

☐ was filed as PCT International Application No. PCT/______________________ on ______________________

and was amended on ______________________ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulation, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

(Number)	(Country)	(Day/Month/Year Filed)	Priority claimed
			☐ yes ☐ no
			☐ yes ☐ no

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulation, Section 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Application Number)	(Filing Date)	(Status- patented, pending, abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: John F. Moran, Reg. No. 26,313 and Anthony T. Lane, Reg. No. 19,964.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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☐ Additional inventors are being named on separately numbered sheets attached hereto.

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